Mucosal oxidative injury in murine dextran sulfate-induced colitis

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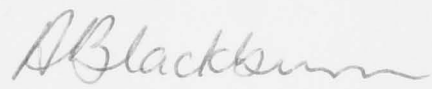
Except where otherwise indicated, this thesis is my own original work.



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Abstract

Reactive oxygen and nitrogen species (RONS) have been implicated as mediators of mucosal injury in inflammatory bowel disease (IBD), however, the oxidative modifications which may be occurring in the inflamed tissue have not been identified. The aim of this study was to investigate the molecular nature of the oxidative tissue injury in acute colitis induced in mice by oral 5% dextran sulfate (DSS). Mucosa collected from DSS-exposed mice was compared to mucosa from control mice for a variety of indicators of oxidative injury which explored the similarities of this model to the human disease in terms of what is already known about the oxidative changes in the mucosa from IBD patients. New markers of oxidative injury were also investigated in DSS-induced colitis and IBD.

To determine whether oxidant production may be increased in the inflamed mucosa of DSS-induced colitis, assessment was made of mucosal myeloperoxidase (MPO) activity and hydroxyl radical ($^{\bullet}OH$) generation. Compared to control mucosa, there was a three to ten-fold increase in mucosal MPO activity ($P < 0.001$), corresponding to the neutrophil infiltration seen histologically. $^{\bullet}OH$ generation was assessed by measuring the $^{\bullet}OH$-specific product of salicylate hydroxylation, 2,3-dihydroxybenzoic acid (2,3-DHB). While salicylate hydroxylation studies revealed an 83% increase in 2,3-DHB levels, they also revealed a 34% decrease in the ratio of 2,3-DHB to salicylate in inflamed mucosa, possibly indicating greater catabolism or decreased production of 2,3-DHB. Thus it was not clear whether the amount of $^{\bullet}OH$ generated had decreased, or if other metabolic changes were obscuring the measurement of $^{\bullet}OH$ by this method. While hypochlorous acid (HOCl) could oxidise 2,3-DHB *in vitro*, HOCl did not appear to be involved in catabolism of DHB *in vivo* in DSS-induced colitis.

To explore biochemical changes in the mucosa which may be due to oxidative injury, the status of the major non-enzymic antioxidants and thiol indicators in the normal and inflamed mucosa were investigated. There was a significant 16% decrease in total scavenging capacity ($P < 0.05$) and significant changes in mucosal antioxidant levels, including depletion of ubiquinol-9 and ascorbate by 53% and 20% respectively ($P < 0.001$) and elevation of α-tocopherol and urate levels by 64% and 109% respectively ($P < 0.001$). Glyceraldehyde-3-phosphate dehydrogenase activity (inactivated by thiol oxidation in inflamed but not non-inflamed IBD epithelium) and total reduced thiol content were also significantly decreased by 34% and 26% respectively ($P < 0.001$). These results support a role for excessive oxidant production and thiol oxidation in the tissue injury of this disease. The changes observed closely resembled those reported in human IBD tissue, indicating that DSS-induced colitis is a highly suitable model for studying further mechanisms of oxidative tissue injury in IBD.

To further explore protein oxidation in colitis, protein carbonyls were investigated.

Carbonyls were derivatised with dinitrophenyl (DNP) hydrazine for spectrophotometric quantitation and identification of carbonyl-modified proteins by western blotting with anti-DNP probing. Analysis of protein carbonyls in mouse colonic mucosal homogenates oxidised *in vitro* indicated carbonyl formation occurred after HOCl, nitric oxide ($^{\bullet}NO$) and iron-mediated oxidation. Many proteins, including those with molecular weights 47, 75–110, 116, 131, 142 kDa exhibited increased carbonyl content. While protein carbonyl content of inflamed mouse mucosa was not significantly different from control mucosa, western blotting analysis indicated several proteins of molecular weight 48, 79, 95 and 131 kDa, corresponding to those observed after *in vitro* oxidation, which may be particular targets for carbonyl oxidation. Quantitation of the anti-DNP signal from 9 IBD biopsy pairs showed no pattern of protein oxidation in inflamed mucosa, with inflamed/non-inflamed signal ratio >1 in 4 pairs, <1 in 4 pairs, and $=1$ in 1 pair. However, the human tissue showed several candidate proteins of similar molecular weight to those observed in the inflamed mouse mucosa. Identification of the mucosal proteins susceptible to carbonyl-modification and analysis of their activity in IBD may offer insights into protein oxidation and the malfunction of the colonic mucosa.

Considerable indirect evidence has been reported suggesting a role for $^{\bullet}NO$ in the pathogenesis of IBD. The role of $^{\bullet}NO$ in colitis was investigated by examining the effect on disease course of the nitric oxide synthase inhibitor nitro-L-arginine methyl ester (L-NAME), and by investigating nitration of mucosal proteins by western blotting using anti-nitrotyrosine antibodies. Preliminary results with L-NAME treatment of mice did not improve disease symptoms, suggesting that excessive $^{\bullet}NO$ production does not play a major role in the pathogenesis of DSS-induced colitis. Nitrotyrosine analysis of mucosal proteins detected several proteins, but no marked difference was observed between inflamed and non-inflamed tissue from DSS-exposed mice, or from paired IBD biopsies. The contrast between these findings and other models of intestinal inflammation reflect the complex role of $^{\bullet}NO$ in intestinal function, which is yet to be fully elucidated.

The changes MPO activity, antioxidant levels and thiol markers in the mucosa of DSS-induced colitis and their similarity to the human disease suggest this model is highly relevant to the study of oxidative tissue injury in IBD. The other markers of oxidative injury investigated were indicative of the difficulty, in terms of specificity and stability of markers and the distribution of colitis within the tissue, of studying oxidative injury in disease. The application of further specific and *in situ* techniques for the detection of oxidants and tissue injury products offer much for the future of investigations into the role of oxidative tissue injury in the pathogenesis of IBD.

Publications

Papers

The work from Chapter 4 has been submitted (July 1996) for publication in Laboratory Investigation under the title *Colonic antioxidant status in dextran sulfate-induced colitis in mice*.

Meetings

The work from Chapter 4 was presented at meetings and published in abstracts as follows:

Blackburn AC, Buffinton GD, Doe WF. Oxidative tissue injury in dextran-sulphate colitis in mice. Journal of Gastroenterology and Hepatology. 1994;9(suppl):A106

Blackburn AC, Buffinton GD, Doe WF. Oxidative injury to colonic mucosa in dextran sulphate (DSS)-induced colitis. Procedings of the 7th biennial scientific meeting of the International Society for Free Radical Research. 1994;P3

Blackburn AC, Buffinton GD, Doe WF. Oxidative injury to colonic mucosa in dextran sulfate (DSS)-induced colitis in mice. Gastroenterology. 1995;108(suppl): A784.

The work from Chapter 3 and 5 was presented at meetings and published in abstracts as follows:

Blackburn AC, Buffinton GD, Doe WF. Iron mediated mucosal tissue injury in dextran sulfate-induced colitis in mice. Journal of Gastroenterology and Hepatology. 1995;10(suppl):A.

Blackburn AC, Buffinton GD, Doe WF. Iron mediated mucosal tissue injury in dextran sulfate-induced colitis in mice. Proceedings of the 5th regional meeting of the Society for Free Radical Research (Australasia). 1995.

Blackburn AC, Buffinton GD, Doe WF. Carbonyl oxidation of mucosal proteins in dextran sulfate-induced colitis in mice. Gastroenterology. 1996;110(suppl): A866.

Abbreviations

5-ASA	5-Aminosalicylic acid
[^{14}C]-IAM	[^{14}C]-Iodoacetamide
AAPH	2,2′-Azobis(2-amidinopropane) dihydrochloride
BDH	British Drug House
BSA	Bovine serum albumin
BSO	Buthionine sulfoximine
CD	Crohn's disease
cNOS	Constitutive NOS
DHB	Dihydroxybenzoate
DNP	Dinitrophenyl
DNPH	Dinitrophenylhydrazine
DSS	Dextran sulfate sodium
DTNB	5,5′-dithiobis(2-nitrobenzoic acid)
EC	Electrochemical
ESR	Electron spin resonance spectroscopy
fMLP	Formylmethionyl-leucyl-phenylalanine
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GSH	Glutathione
GSSG	Glutathione disulfide
HBSS	Hank's balanced salt solution
HPLC	High performance liquid chromatography
IBD	Inflammatory bowel disease
iNOS	Inducible NOS
L-NAME	Nitro-L-arginine methyl ester
LPS	Lipopolysaccharide
MCO	Metal-catalysed oxidation
MPO	Myeloperoxidase
NOS	Nitric oxide synthase
PBS	Phosphate-buffered saline
PG-PS	Peptidoglycan-polysaccharide
PMSF	Phenylmethylsulfonyl fluoride
RONS	Reactive oxygen and nitrogen species
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS polyacrylamide gel electrophoresis

SOD	Superoxide dismutase
TBS	Tris-buffered saline
TCA	Trichloroacetic acid
TNB	5-Thio-2-nitrobenzoic acid
TNBS	2,4,6-Trinitrobenzene sulfonic acid
UC	Ulcerative colitis

Contents

Chapter 1

Reactive Oxygen and Nitrogen Species in Intestinal Inflammation

1.1 Inflammatory Bowel Disease

Inflammatory bowel disease (IBD) is a collective term for the disorders of ulcerative colitis (UC) and Crohn's disease (CD) which are characterised by chronic, relapsing inflammation of the gastrointestinal tract. The cause of these diseases is not known, although the understanding of factors involved in their pathogenesis is increasing. These diseases are often considered together because of the clinical similarities, the similar approach to management, and the likelihood of common pathophysiologic processes, however there are differences in their characteristics and possibly different initiating events.[1]

The clinical features of IBD include diarrhoea, bloody stools, cramping abdominal pain, and when colitis is extensive, weight loss and anaemia. It is difficult to distinguish between UC and CD on clinical signs, with further diagnosis reliant on differences in the macroscopic and microscopic features of disease.

1.1.1 Epidemiology and Aetiology

IBD is most prevalent in western societies while it is rare in developing countries, with the highest annual incidence rates coming from regions such as Scandinavia and Great Britain: 14–15 new cases per 100,000 population per year for UC; 6–8 per 100,000 per year for CD.[2] These figures are representative of several other European and western countries where studies have been performed, and the temporal trend is towards increasing incidence of CD, although the incidence for UC is relatively stable.[1,2] IBD occurs in people of all ages, with the most common age of onset being 15 to 30 year age group, with a small secondary increase after 55 years.[3] There appears to be a male predominance in UC, and female predominance among CD patients, but this may vary when looking at age-specific incidence rates.[2]

The aetiology of IBD is unknown with no infectious agent being found. Evidence clearly supports both a genetic and environmental component in the susceptibility of an individual to developing IBD. Racial and ethnic differences in incidence suggest a

genetic component, with the incidence highest among Caucasians, and lower in blacks and asians, and some racial groups, such as Ashkenazi Jews, having strikingly higher prevalence.[4,5] Familial aggregation of both diseases, with 15–30-fold increased risk of developing the disease among siblings, further suggests a genetic component.[6,7] Concordance rates among monozygotic twins of 58.3% for Crohn's disease and only 6.3% for ulcerative colitis suggest a strong a genetic component to CD, while environmental factors may be a stronger influence in UC.[8] Urban populations consistently have a higher incidence of both UC and CD than rural populations,[9–11] and smoking has also been identified as an influential environmental factor, with smokers at lower risk of developing UC, but double the risk of acquiring CD.[12]

1.1.2 Pathology of IBD

There are several structural and functional features of the normal human colon (Figure 1.1) which are involved to varying extents and assist in the differentiation of CD and UC. The mucosa, the inner most layer, consists of the surface epithelium layer, be-

Figure 1.1: Histology of normal human colonic mucosa. Crypt structures of epithelial and goblet cells are surrounded by lamina propria.

neath which are crypt structures of epithelial and goblet cells. The crypts are responsible for the absorptive capacity of the colon and the goblet cells secrete muc us to provide a protective layer between the surface epithelium and the lumen. The crypts are surrounded by the lamina propria, a network of fibroblasts, extracellular matrix and resident macrophages. The muscularis mucosa separates the mucosa from the submucosa, which lies above the muscularis externae, the large outer layer of muscle which is responsible for the peristaltic motions of the colon.

Macroscopically, UC invariably involves the rectum and extends proximally in a continuous manner, but is restricted to the colon (Figure 1.2). Colon shortening occurs as a result of muscle contracture and thickening, and pseudopolyps are common. Inflammation is confined to the mucosa and submucosa, with congestion of vessels,

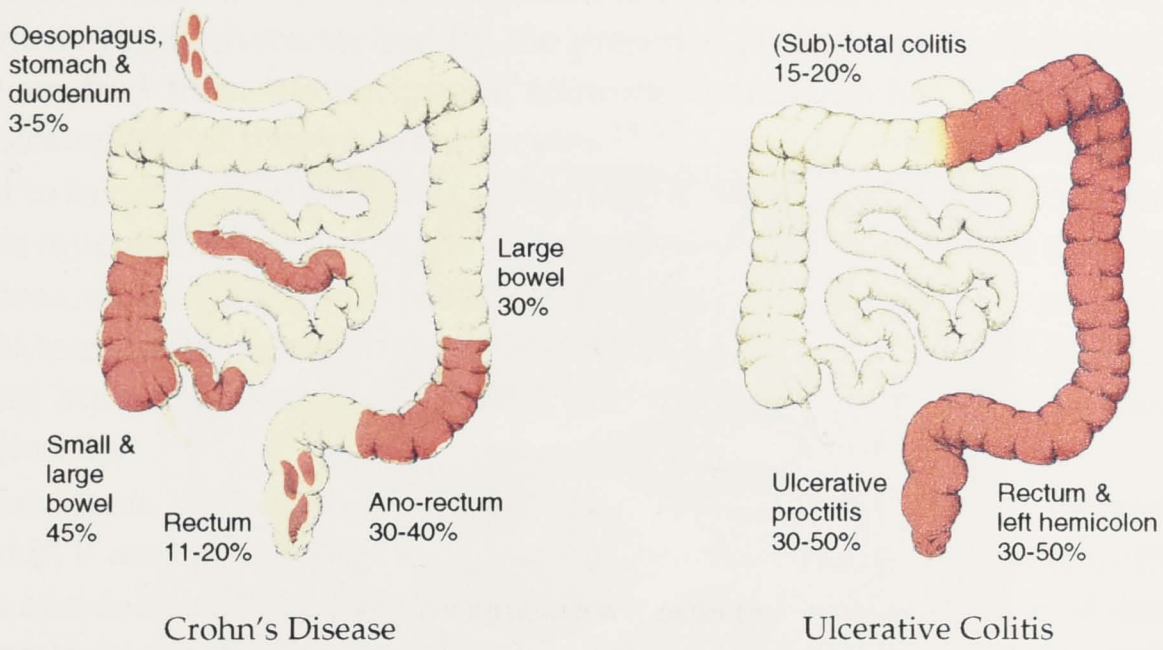


Figure 1.2: Relative localisation of Crohn's disease and ulcerative colitis in the alimentary tract. (Adapted from Dr. Falk Pharma GmbH, Scheme A10 (1989) and A11 (1991) posters by E. Dirks.)

intramucosal haemorrhage and oedema in active colitis. The lamina propria is infiltrated by inflammatory cells, with foci of neutrophils and the formation of crypt abscesses common (Figure 1.3). Goblet cells are depleted of mucin, and loss of goblet cells also occurs.[1,13] In long standing, chronic disease, there is a significant increase in the risk of developing colon cancer, which may be as high as 15–19-fold in patients with extensive colitis.[14,15]

By comparison, CD can involve any part of the alimentary tract, with the rectum and terminal ileum being most commonly affected (Figure 1.2). The inflammation is often discontinuous, with involved areas being separated by normal tissue. Macroscopically, the inflamed mucosa has a cobblestoned appearance, due to deep ulceration and fissure formation characteristic of CD. Transmural inflammation of the intestinal wall and the presence of granulomas in many CD patients also distinguish CD from UC, where the inflammation is confined to the mucosa and granulomas are absent (Figure 1.4).[1,13]

1.1.3 Mechanisms of Tissue Injury

It is generally accepted that the immune system is primarily responsible for the mucosal damage seen in these pathologies. Although the initiating events are not known, there is sufficient evidence to construct an explanatory scheme for the chronic, persistent nature of the inflammation of IBD.

The mucosal immune system differs considerably from the peripheral immune system. Lipopolysaccharide (LPS), a major product of Gram-negative bacteria cell walls, is present in abundance in the lumen of the intestine. Although LPS is a potent stimulus for a variety of receptor-bearing immune cells, resident macrophages in

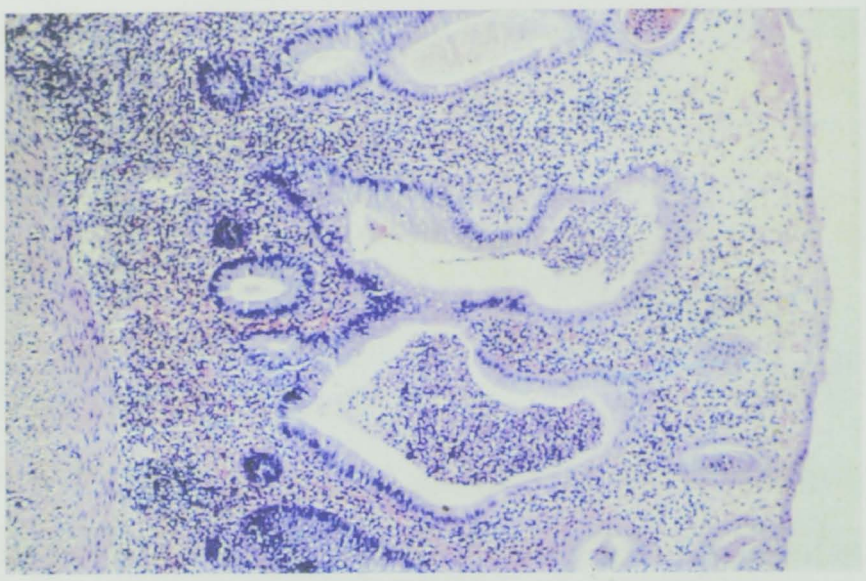


Figure 1.3: Histological features of UC: the lamina propria is infiltrated predominantly with neutrophils, and crypt abscesses are found.

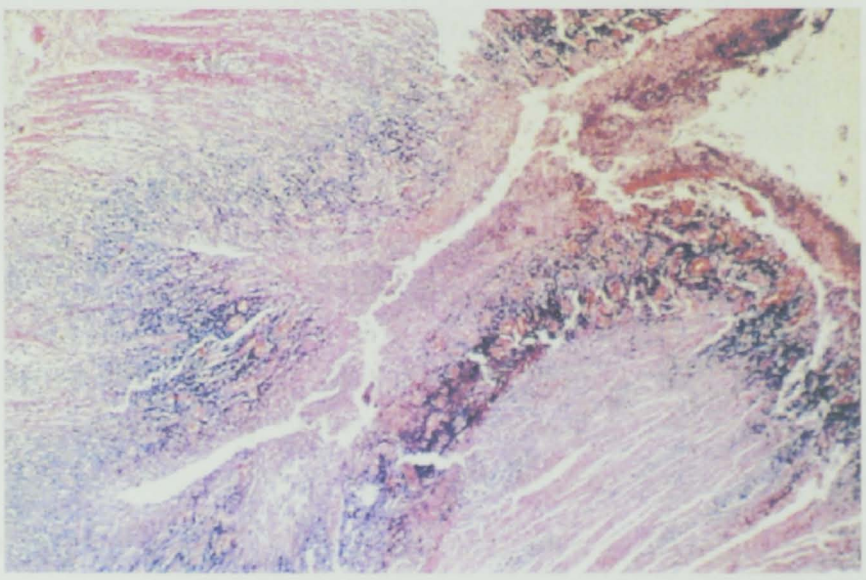


Figure 1.4: Histological features of CD: marked submucosal inflammatory infiltrate and a deep fissure are displayed.

the normal intestinal mucosa are tolerant to stimulation by LPS.[16,17] The active IBD lesion, however, is characterised by the presence of neutrophils and macrophages recently recruited from the peripheral immune system, as has been demonstrated by the radiolabelling of patient granulocytes.[18–20] Considerable research effort has been directed to investigating the factors which attract and activate these cells in IBD as, unlike their mucosal counterparts, these cells are highly responsive to the LPS present in the mucosa, resulting in the production of many pro-inflammatory mediators, including cytokines, leukotrienes, prostaglandins and oxidants, many of which are chemotactic and attract further immune cells into the lesion, or which may mediate tissue injury directly.

Tissue levels of the pro-inflammatory cytokines interleukin 1, interleukin 6 and interleukin 8 are consistently increased in actively inflamed mucosa in IBD.[21] Interleukin 1 and interleukin 6 stimulate the activation and proliferation of resting T cells, which synthesise additional cytokines to further amplify the inflammatory response, while interleukin 8 is a potent chemoattractant for neutrophils.[22,23] There is also good evidence for elevated production of eicosanoids in IBD,[22] with leukotrienes, products of the enzyme 5-lipoxygenase, considered the most important pro-inflammatory eicosanoids.[24] In particular, leukotriene B_4 is present at 10-fold control levels in IBD mucosa and accounts for the major part of the chemotactic activity of IBD mucosal homogenates.[25,26]

Cytokines, leukotrienes and LPS may have direct consequences for epithelial cell viability, proliferation and function, however, most of the tissue damage in acute IBD is considered to be mediated by macrophages, neutrophils and eosinophils attracted and stimulated by the inflammatory mediators.[21,27] Macrophages and neutrophils exert their destructive potential through the secretion of multiple oxidants and proteases (Section 1.2.3),[28,29] and many studies support a role for oxidants in the tissue injury of IBD (Section 1.3). The resulting tissue damage exposes the mucosa to further LPS and luminal antigens, with the stimulation of further cytokine production and chemoattraction, completing the cycle for the perpetuation of mucosal inflammation.

1.1.4 IBD Therapies

The principal agents of medical therapy for IBD have remained unchanged since the 1960s, with development of new anologues or drug delivery accounting for most of the advances. There are three broad categories of drug therapies for IBD, aminosalicylates, steroids and immunosupressives, which may be considered in terms of the severity of disease for which they are used.[30–32] These treatments remain non-specific and their mechanisms of action are not fully understood, although the efficacy of these therapies in IBD can be explained partially in terms of modulation of the inflammatory mediators described in Section 1.1.3.

Aminosalicylates, including sulfasalazine and olsalazine, are the primary treatment for mild to moderately active disease in both UC and CD. They can be administered as enemas, or orally for long term treatment, where benefits are also found in the maintenance of remission in UC, although this is not established in CD. The active

moiety of these treatments is 5-aminosalicylic acid (5-ASA) which, in sulfasalazine and olsalazine, is covalently bound to a sulfapyridine or 5-ASA molecule respectively by an azo-bond (Figure 1.5). The azo linkage is cleaved by intestinal bacteria to release 5-ASA, allowing high concentrations to be achieved in the colon while avoiding small intestinal absorption and consequent side effects and metabolism.

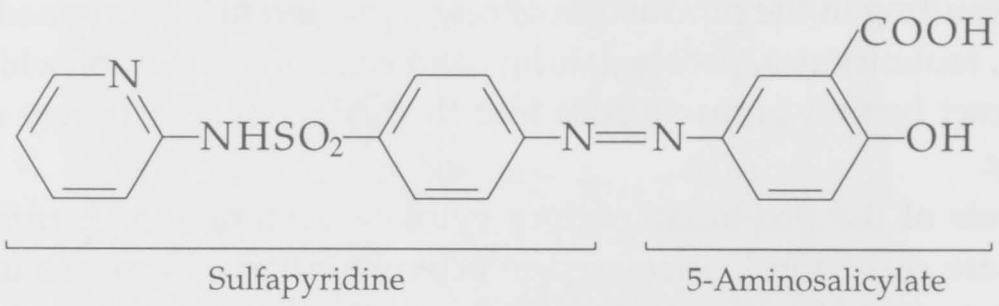


Figure 1.5: Sulfasalazine consists of sulfapyridine and 5-ASA joined by an azo bond. Bacterial cleavage of the azo linkage releases the active moiety, 5-ASA, in the proximal colon.

The precise mechanism of action of aminosalicylates remains unknown. The activities of these compounds which may contribute to their efficacy in IBD are many, reviewed in detail by Travis and Jewell.[33] They include inhibition of the enzyme 5-lipoxygenase and the synthesis of leukotrienes, protection against increases in mucosal permeability, inhibition of chemotaxis of macrophages and inhibiton of cytokine synthesis and release by monocytes. 5-ASA also has considerable antioxidant properties, such as the inhibition of oxidant generation and direct scavenging of oxidants (Section 1.3.4).

In IBD patients who do not respond to aminosalicylates, *corticosteroids* are used for moderate-to-severe cases of both CD and UC,[30] however this treatment induces remission in only about 50% of patients with active disease, and is not useful in maintaining remission. Oral prednisone or intravenous methylprednisolone are useful in cases too severe or unresponsive to aminosalicylates. Topical application of steroids is also used in distal disease, although as much as 20% of the dose can be absorbed, leading to systemic side effects. In recent years, more effective topical steroids have been trialled, such as budesonide and beclomethasone, which undergo extensive first-pass metabolism in the liver, reducing the likelihood of systemic side effects.[34–36]

Immunosupressive agents are also proving useful in the management of IBD. Azathioprine and its active derivative, 6-mercaptopurine, are useful in maintaining remission in patients with CD, where neither sulfasalazine or corticosteroids are effective. Cyclosporin has also been evaluated in several trials, and appears useful for short term therapy of severe cases of UC[37] but its value in longer term therapy must be carefully considered against possible side effects.[30]

While these therapies are useful for controlling disease, over a lifetime a high proportion of patients will suffer uncontrollable disease, requiring surgical removal of the affected portion of intestine. After 20 years of UC, colectomy rates are estimated at 15–40%, and for CD, 56% of patients in one study required surgery over a period

of 10 years, with 18% requiring further surgery.[3] Potential new therapies for IBD are appearing, based on our present understanding of the pathology of the disease. These include selective 5-lipoxygenase inhibitors,[24,38] which reduce the production of leukotrienes such as leukotriene B_4, hydroxychloroquine, which slows antigen presentation by epithelial cells,[30,31] the radical scavenger superoxide dismutase[39] (Section 1.3.4) and anti-tumour necrosis factor antibody.[40] The limited trials of these novel agents are indicative of the need for greater understanding of the complex inflammatory processes underlying IBD so that further therapeutic approaches can be developed, even if the initiating pathogenetic events remain elusive.[30]

1.1.5 Animal Models of IBD

The limited understanding of the pathogenesis of IBD has been contributed to by the absence of good animal models of these diseases.[1] The ideal animal model would be similar to the human disease in its clinical course, therapeutic response, histopathology and inflammatory mediator profile. Features such as chronic disease and the rela psing nature of the human disease have been particularly hard to mimic. Additional considerations are expense, convenience, timecourse, availability of the animal strain, availability of molecular probes and antibodies, and reproducibility. There are many models of colitis available which have contributed significantly to the understanding of the pathogenesis of IBD, and have been reviewed recently.[41–43] These models can be divided into two broad categories of spontaneous and induced models of colitis.

1.1.5.1 Spontaneous Models of Colitis

Spontaneous models, while more difficult to work with, provide valuable insights into the initiating events of this disease and the genetic and environmental factors influencing the predisposition of animals to develop disease.

The *cotton-top tamarin*, when held in colonies in temperate climates, develops a disease remarkably similar to UC. The disease is spontaneous and undergoes relapses and remissions, is associated with colon cancer, and the acute disease responds to treatment with sulfasalazine.[44,45] A similar condition has also been found in a captive *rhesus macaque* colony.[46] The limited availability and expense of these species has restricted the number of studies performed on these models, however, they have been able to supply information on the effects of diet, environment and transmissable agents on the occurrence of colitis and cancer in cotton-top tamarins[47] and on the involvement of nitric oxide in idiopathic, spontaneous colitis.[48]

The development of spontaneous rodent models has made spontaneous colitis a much more accessible tool. Recently a substrain of mice, *C3H/HeJBir*, was established which developed spontaneous, heritable colitis.[49] The disease onset coincides with bacterial colonisation of the gut, however C3H/HeJ mouse strains are not sensitive to LPS, and the inflammation is usually mild and resolves itself by 10–12 weeks of age. The genetic defect in these mice is yet to be identified.

Other spontaneous models are appearing in the form of *knockout mice*. Knockout mice currently reported to display intestinal inflammation include interleukin 2, interleukin 10, transforming growth factor β1, T cell receptor α or β, and the G protein subunit $G\alpha_{i2}$.[43,50] These diseases generally include chronic inflammation of the colon which is unresolving and not relapsing, and are indicative of the wide range of immunoregulatory defects which can lead to chronic colitis and potentially contribute to IBD.

1.1.5.2 Induced Colitis

While spontaneous models are important for understanding the initiating events in colitis, the later events in the disease process are more easily studied when the disease is induced in a controlled and predictable manner. While the initiating events in these models may not be comparable to the human condition, the processes and inflammation that proceed initial injury to the mucosa may be of great relevance to the pathogenesis of IBD.

The induction procedures and agents vary enormously, from the luminal instillation of chemicals such as acetic acid, to the intramural injection of peptidoglycan-polysaccharide polymers, resulting in models with different characteristics which have been reviewed extensively.[41–43] The following is a brief description of those models which have been used in studies on the role of oxidants in colitis, as discussed in Section 1.3 and throughout this thesis.

Acetic acid-induced colitis has been described in rats, mice, guinea pigs and rabbits. Colitis is induced by luminal instillation of acetic acid (4–10%) for 15–30 sec. Direct toxicity to the epithelium of this organic acid is understood to be the initiating event, with luminal factors and arachidonic acid pathways amplifying the inflammation. The inflammation is acute, has a similar inflammatory mediator profile to IBD[51] and is responsive to IBD therapies. The lack of chronicity is considered a problem with this model.

2,4,6-Trinitrobenzene sulfonic acid (TNBS) *-induced inflammation* is a widely used model, with inflammation induced by intraluminal instillation of TNBS in ethanol. This produces acute and chronic inflammation, which is transmural with occasional granulomas, suggesting similarities to CD. The mechanism of inflammation is thought to be through macrophage recognition of TNBS-modified proteins, and through metabolism of TNBS by the mucosa to produce reactive oxygen species.[52]

Luminal perfusion of the terminal ileum with the bacterial peptide *formylmethionyl-leucyl-phenylalanine* (fMLP) induces acute intestinal inflammation. Changes in the intestine, such as increases in mucosal and microvascular permeability, are attenuated by sulfasalazine and appear to be mediated largely by activated neutrophils attracted to the mucosa. This model has been used for studies of oxidant-mediated injury by neutrophils.[53–56]

Continuous oral administration of low molecular weight *carrageenan* induces mucosal inflammation of the caecum and colon in 5–7 days. This is thought to be due to increased mucosal permeability due to destruction of intraepithelial tight junctions.

Luminal bacteria have a well documented role in this disease, but the expense of and variability in carrageenan supplies make this model impractical for drug screening.[43]

Peptidoglycan-polysaccharide (PG-PS) *enterocolitis* is induced by the intramural injection of PG-PS polymer into multiple sites in the distal ileum or colon of the rat. This results in an acute inflammation several days after injection, followed by a chronic inflammation at 3–4 weeks. Intestinal inflammation is discontinuous, with granulomas and possibly transmural lesions, thus largely resembling CD.[57,58] While this is an attractive chronic model, with inflammation persisting as long as 6 months in some rat strains, the difficulty of effectively administering the PG-PS preparation remains the major drawback.

A single intraperitoneal injection of *mitomycin C* induces a transient, diffuse colonic inflammation in rats which, like UC, is confined to the mucosa. Again, the mechanism of induction is not understood, but it appears that luminal bacteria may not be essential, that increases in permeability may be the result rather than a cause of inflammation, and that oxidants, possibly generated by mitomycin C, play a role.[59]

The lack of a convenient chronic, relapsing model of induced colitis that was both economic and reproducible has lead to the characterisation of the acute and chronic *dextran sulfate sodium* (DSS) *models of colitis*[60,61] described in detail in Chapter 2. Acute colitis is induced by administering high molecular weight, highly sulfated dextran sulfate in the drinking water of mice for 7 to 10 days, which if followed by 7 to 14 days of water, becomes chronic. This disease is confined to the colon, appearing as multiple erosive lesions with inflammatory cell infiltration, including polymorphonuclear leucocytes, however few crypt abscesses and granulomas have been observed. While the mechanism of induction of this disease is not known, the current understanding is described in Chapter 2. The distinct advantages of this model over other models is its simplicity of induction, the ability for both a chronic or acute disease to be induced, and the uniformity and reproducibility of disease within a given strain of animal.[62] This model is an appropriate one for studying contributions from the innate immune system and nonspecific inflammatory processes, such as those involving activated phagocytes, and for drug screening studies.[43]

1.2 Free Radical Biochemistry

1.2.1 Reactive Oxygen and Nitrogen Species

Oxygen, while being essential for many forms of life, is potentially very toxic. A free radical is a molecule with one or more unpaired electrons which, since electrons are generally more stable when paired, makes them more reactive than non-radicals. Examples of radicals of interest in biological systems are superoxide ($O_2^{\bullet -}$) and hydroxyl radical ($^{\bullet}OH$). These radicals can undergo direct reactions with biomolecules, or may be transformed into other non-radical yet potent oxidants, such as hydrogen peroxide (H_2O_2) and hypochlorous acid ($HOCl$). Acknowledgement of the role of nitrogen oxides, such as nitric oxide ($^{\bullet}NO$) and peroxynitrite ($ONOO^-$) in biological systems has lead to the phrase "reactive oxygen and nitrogen species" (RONS) being used to

collectively refer to the various radicals of oxygen and nitrogen, and other oxidative species involved in biological processes.

While oxidants were once considered as by-products of metabolic reactions, increasing numbers of physiological processes in which RONS are involved are coming to light. Perhaps the longest recognised function of oxidants is in the killing potential of phagocytes, where oxidants are produced at high concentrations within the phagosome for destruction of foreign material and invading microbes (Section 1.2.3). More recently, roles for RONS produced constitutively at low levels are being recognised. Low concentrations of RONS such as H_2O_2, $O_2^{\cdot-}$ and $^{\cdot}NO$ have been found to stimulate proliferation of cells such as lymphocytes and fibroblasts.[63,64] $^{\cdot}NO$ is involved in many aspects of physiology including the vascular system, as a vasodilator produced by endothelial cells,[65,66] and the central nervous system, as a mediator of neuronal responses to excitatory amino acids.[66] A role for oxidants in intracellular signalling pathways involving nuclear factor κB and activator protein 1 has been identified.[67] The production and reactions of RONS in biological systems, with particular attention to their relevance in inflammatory disease, are outlined in this section.

1.2.1.1 Production of Reactive Oxygen Species

Reactive oxygen species are produced continuously as normal byproducts of cellular metabolism, and cells have appropriate mechanisms for detoxifying them and thus preventing damage to tissues. The mitochondrial electron transport chain is one of the most important sources of $O_2^{\cdot-}$ in most aerobic cells. While electrons are passed down the chain from reducing equivalents NADH and FADH for conversion of O_2 to H_2O and the generation of ATP, some components of the chain "leak" electrons to molecular oxygen to form $O_2^{\cdot-}$. Some 1–5% of the oxygen consumed in respiration is converted to $O_2^{\cdot-}$,[68] which while itself is not highly toxic, is precursor to a host of more potent oxidants.

Two molecules of superoxide will react, either spontaneously or catalysed by the antioxidant enzyme, superoxide dismutase, to produce hydrogen peroxide:

$$2O_2^{\cdot-} + 2H^+ \longrightarrow H_2O_2 + O_2 \tag{1.1}$$

H_2O_2 is a relatively long-lived, weak oxidising agent capable of inactivating some enzymes by oxidation of essential thiols. Unlike the charged $O_2^{\cdot-}$, H_2O_2 is a membrane permeable species, and once inside the cell may react with Fe^{2+} to mediate toxicity. $O_2^{\cdot-}$ can also reduce transition metal ions such as iron, in a one electron transfer:

$$O_2^{\cdot-} + Fe^{3+} \longrightarrow O_2 + Fe^{2+} \tag{1.2}$$

Ferrous ions are then able to participate in the decomposition of H_2O_2 to form the highly toxic hydroxyl radical in the Fenton reaction:

$$Fe^{2+} + H_2O_2 \longrightarrow {}^{\cdot}OH + OH^- + Fe^{3+} \tag{1.3}$$

The net result of equations 1.2 and 1.3 is the iron-catalyzed Haber-Weiss reaction:

$$O_2^{\cdot-} + H_2O_2 \xrightarrow{Fe^{3+}} {}^{\cdot}OH + OH^- + O_2 \tag{1.4}$$

In contrast to H_2O_2 and $O_2^{\cdot-}$, $^{\cdot}OH$ reacts at close to a diffusion-controlled rate with all biological molecules, and its half-life in cells has been estimated to be 10^{-9} seconds. Because of its extreme reactivity, it has been thought to be responsible for much of the toxicity of superoxide, although the availability of iron in a form able to catalyse this reaction in normal tissues has always been an area of contention (Section 1.2.2.3).[69]

In addition to mitochondrial "leakage", $O_2^{\cdot-}$ is produced by several enzymes. Phagocytic leukocytes possess an enzyme called NADPH-oxidase, present in the cell membrane, which releases $O_2^{\cdot-}$ into the phagosome as a precursor to other potent oxidants (Section 1.2.3). This is central to the microbicidal activity of these cells.

Xanthine oxidase produces $O_2^{\cdot-}$ as it catalyses the oxidation of hypoxanthine to xanthine, and of xanthine to uric acid. In most tissues, these reactions are largely catalysed by xanthine dehydrogenase (XD), which concommittantly converts NAD^+ to NADH. However, oxidation of thiol groups or limited proteolysis can convert xanthine hydrogenase to xanthine oxidase (XO), which uses molecular oxygen as its electron acceptor, and so produces $O_2^{\cdot-}$:

$$\text{xanthine} + NAD^+ + H_2O \xrightarrow{XD} \text{uric acid} + NADH + H^+ \tag{1.5}$$

$$\text{xanthine} + 2O_2 + H_2O \xrightarrow{XO} \text{uric acid} + 2O_2^{\cdot-} + 2H^+ \tag{1.6}$$

This enzyme conversion step occurs during periods of ischaemia, and the consequent production of $O_2^{\cdot-}$ upon re-oxygenation has been implicated in the tissue damage of ischaemia/reperfusion injury.[70]

Other enzymes which can produce radicals are cyclooxygenase and lipoxygenase in the metabolism of arachidonic acid into prostaglandins and leukotrienes respectively. In these metabolic pathways, there are several radical and peroxide intermediates formed which can initiate lipid peroxidation chain reactions (Section 1.2.4).

1.2.1.2 Production of Reactive Nitrogen Species

Another physiological free radical is $^{\cdot}NO$, first identified as endothelium-derived relaxing factor (EDRF).[65] It is produced by a class of enzymes called nitric oxide synthases (NOS) which catalyse the deamination of arginine to citrulline:

$$\text{arginine} + NADPH + O_2 \longrightarrow \text{citrulline} + NADP^+ + {}^{\cdot}NO \tag{1.7}$$

The characteristics of the enzyme activity and the cofactor requirement for this reaction depend on the isoform in question. There are two main groups of NOS, constitutive and inducible.

Constitutive isoforms (cNOS) are dependent on calcium/calmodulin binding for activity and produce picomoles of $^{\cdot}NO$ for short periods of time. Purified largely from endothelial cells (ecNOS) and neurones (nNOS), constitutive NOSs are involved

in important functions in the regulation of vascular tone and neurotransmission.[66] Inducible isoforms (iNOS) are calcium/calmodulin independent, and FMN and FAD dependent. First identified in macrophages, iNOS gene expression and activity is induced by certain cytokines and bacterial products such as interferon γ, LPS and fMLP to produce nanomoles of $^{\bullet}NO$ for longer periods of time, in the cytotoxic activity of macrophages. It is now appreciated that many cell types express cNOS and iNOS in physiological and disease situations.[66,71,72]

$^{\bullet}NO$ is a relatively stable radical and sufficiently non-polar to diffuse freely through membranes. It is capable of oxidising thiols to form *S*-nitrosothiols (RS-NO),* and reacting with haem groups and iron-sulfur clusters, reactions which are important in it physiological roles.[66,71] However, in the presence of molecular oxygen, $^{\bullet}NO$ will react to yield nitrogen dioxide ($^{\bullet}NO_2$), which further decomposes to produce nitrite (NO_2^-) and nitrate (NO_3^-):

$$2^{\bullet}NO + O_2 \longrightarrow 2^{\bullet}NO_2 \rightleftharpoons N_2O_4 \xrightarrow{H_2O} NO_2^- + NO_3^- + 2H^+ \qquad (1.8)$$

Nitrates and nitrites are easily measured and are often used as an indirect indicator of $^{\bullet}NO$ production. Alternatively, if $O_2^{\bullet-}$ is also present, then $^{\bullet}NO$ and $O_2^{\bullet-}$ may combine to form the more toxic anion, $ONOO^-$, as described in Section 1.2.3.

In the context of radical injury in disease, iNOS is of most interest because of the quantity of $^{\bullet}NO$ produced and the controls over induction of the enzyme and $^{\bullet}NO$ production by phagocytes (Section 1.2.3). More generally, NOS activity can be inhibited by substrate analogues, which have varying selectivities for the different isoforms, such as aminoguanidine, specific for iNOS,[73] *N*-nitro-L-arginine, relatively specific for cNOS, and *N*-nitro-L-arginine methyl ester, which is relatively non-specific but commonly used.[66]

1.2.2 Antioxidant Protection Mechanisms

While oxidants are always being produced and have roles to play in physiology, excessive production of oxidants or the inappropriate interaction of oxidants with biological molecules may be responsible for tissue injury and the pathology of several disease states (Sections 1.2.4 and 1.2.5). Thus aerobic organisms have evolved with several antioxidant mechanisms for the removal of potentially toxic RONS. Antioxidants have been defined as any substance that, when present at low concentrations compared to that of an oxidisable substrate, significantly delays or prevents oxidation of the substrate.[74] In the biological context, they can be divided into three categories — enzymic antioxidants, chemical or non-enzymic antioxidants and metal-ion binding proteins.

1.2.2.1 Enzymic Antioxidants

Glutathione peroxidase, superoxide dismutase (SOD) and catalase are the most important enzymic antioxidants in mammalian cells. SOD catalyses the dismutation of

*$^{\bullet}NO$ must first interact with an oxidizing centre to give NO^+ before reacting with thiols.

$O_2^{\bullet-}$ to H_2O_2 and molecular oxygen (Equation 1.1). Virtually all eukaryotic cells contain SOD in one or more isoforms — CuZnSOD in the cytoplasm and MgSOD in the mitochondria. The reaction is 10,000 times faster than the spontaneous dismutation of $O_2^{\bullet-}$, and thus $O_2^{\bullet-}$ levels are kept very low.[69]

The H_2O_2 generated by SOD is also removed enzymically by catalase, which catalyses the detoxication of H_2O_2 to water and molecular oxygen:

$$2H_2O_2 \longrightarrow 2H_2O + O_2 \tag{1.9}$$

This enzyme is particularly efficient at high concentrations of H_2O_2 and is located primarily in the peroxisomes of cells. At lower concentrations of H_2O_2, other peroxidases present in the cytoplasm are more important, such as the selenium-containing enzyme glutathione peroxidase, which reduces H_2O_2 at the expense of glutathione (GSH):

$$H_2O_2 + 2GSH \longrightarrow GSSG + 2H_2O \tag{1.10}$$

GSH peroxidase is more versatile than catalase and can also breakdown other peroxides, such as lipid hydroperoxides. To maintain GSH levels, GSH reductase catalyses the reduction of glutathione disulfide (GSSG) back to GSH:

$$GSSG + NADPH + H^+ \longrightarrow 2GSH + NADP^+ \tag{1.11}$$

This requires the reducing equivalent, NADPH, which is supplied by the pentose phosphate pathway. The reduction of GSSG to GSH *via* GSH reductase and NADPH is refered to as the GSH redox cycle, and is very efficent at detoxifying peroxides.[69] The maintenance of reduced GSH levels is also important for other antioxidant mechanisms, such as the reduction of oxidised ascorbate. Thus maintaining adequate levels of reducing equivalents for GSH reductase and other enzymes is important in counteracting oxidant stress.[75,76]

1.2.2.2 Non-enzymic Antioxidants

Chemical antioxidant defences consist of a collection of molecules which reside in the different chemical phases of our cells. These compounds readily scavenge RONS in biological situations, and interact with each other and the enzymic antioxidants in complex ways to prevent tissue injury by RONS (Figure 1.6).

The major antioxidants present in the lipid phase are tocopherol, ubiquinol and β-carotene. α-Tocopherol is present in all membranes and, as a chain-breaking antioxidant, is the primary protection mechanism against lipid peroxidation. α-Tocopherol can be regenerated from the tocopheryl radical by aqueous scavengers such as ascorbate and glutathione. Ubiquinol is present largely in the mitochondrial membranes, and is a component in the electron transport chain. It too is a chain breaking antioxidant and has antioxidant capacities similar to α-tocopherol. β-Carotene, a precursor of vitamin A, can also scavenge various RONS within the lipid phase.[69,77,78]

In the aqueous phase, ascorbate, urate and GSH are important molecules provid-

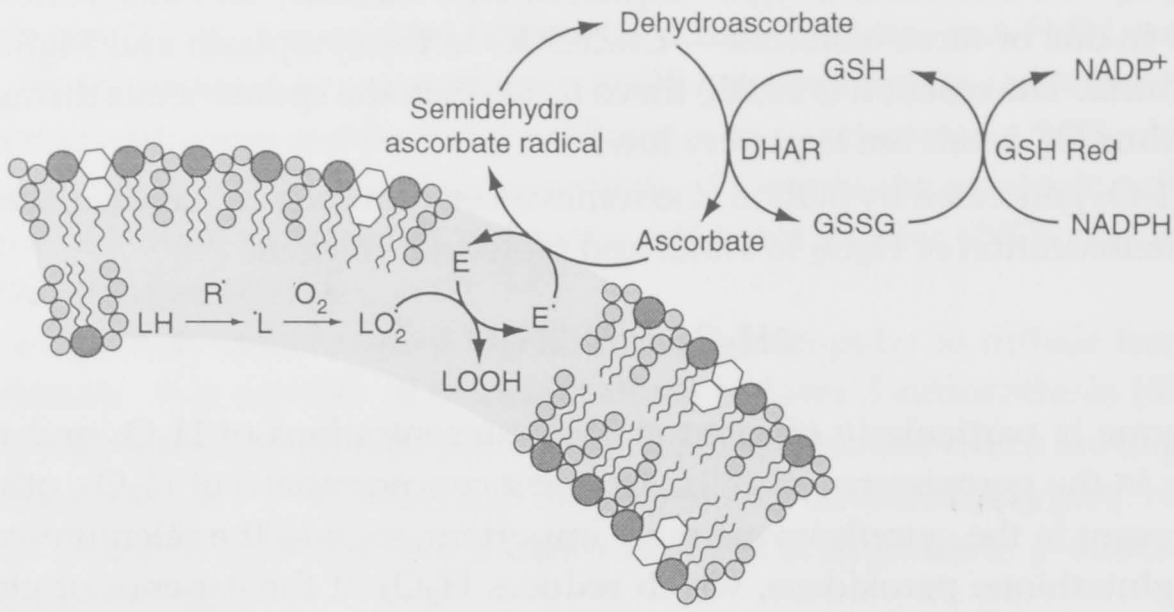


Figure 1.6: Antioxidant mechanisms: the interaction of aqueous, lipophilic and enzymic antioxidants in the prevention of cellular damage by RONS. The components of the membrane represent lipids, cholesterol and tocopherol. L - lipid, E - tocopherol, DHAR - dehydroascorbate reductase, GSH Red - GSH reductase.

ing antioxidant protection. Ascorbate, while present in the aqueous phase, is involved in maintaining the redox status of both aqueous and lipid phases of the cell.[79] Most animals can synthesise ascorbate, but primates and guinea pigs are dependent on dietary intake for maintaining ascorbate levels. It can react directly with many oxidants in the aqueous phase, including $O_2^{\cdot-}$ and $^{\cdot}OH$, but important in its function is its interaction with tocopherol to prevent lipid peroxidation. Ascorbate reduces the tocopheryl radical to tocopherol, allowing it to participate again in its chain breaking antioxidant capacity (Figure 1.6). In scavenging, ascorbate is oxidised to dehydroascorbate, from which reduced ascorbate can be regenerated by a GSH-dependent dehydroascorbate reductase.[80]

Urate has high reactivity to oxygen radicals. An end product of purine metabolism, high concentrations of urate are achieved in biological fluids, such that it accounts for 30-65% of the peroxyl radical scavenging capacity of plasma. It can also complex iron, thus providing further antioxidant protection.[81,82]

Glutathione, a tripeptide thiol, is another important aqueous antioxidant. As well as the reduction of dehydroascorbate and the removal of peroxides by GSH peroxidase, GSH is involved in scavenge radicals directly and plays important roles in maintaining protein thiols and mitochondrial function.[83,84]

1.2.2.3 Metal-ion Binding Proteins

Metal ions such as copper and iron can greatly increase the toxicity of $O_2^{\cdot-}$ and H_2O_2 by catalysing the generation of $^{\cdot}OH$ according to the Fenton reaction (Equation 1.4). The majority of body iron is located in haem-containing proteins. The remaining intra and extracellular iron is bound to the iron storage and transport proteins transferrin, lacto-

ferrin and ferritin, making the iron unable to catalyse such reactions. Transferrin in the plasma is normally only 30% saturated, so that any extra iron released is chelated rapidly. Ferritin binds iron within the tissues, such that it is unable to participate in Fenton chemistry. *In vitro*, copper can also catalyse $^{\bullet}OH$ formation in a similar manner to iron, but *in vivo*, it is bound tightly and inactive in the protein caeruloplasmin.[85,86]

The possibility of a low molecular weight iron pool to catalyse the Fenton reaction has also been suggested. Iron bound to chelators such as citrate and ATP may be available to catalyse the reaction. While numerous investigators have reported finding these intracellularly, the presence of these chelates in the extracellular medium is yet to be confirmed.[85] Thus, in normal tissue, there is little catalytic metal-ion available for catalysis of $^{\bullet}OH$ generation.

1.2.3 RONS Production by Stimulated Inflammatory Cells

The toxicity of RONS is an important component of the microbicidal activity of neutrophils, macrophages and eosinophils. In disease states such as inflammation, it is this source of RONS that is of greatest consequence. The oxidant flux is usually directed at the contents of a phagocytic vacuole, however, in inflammation, tissues also become non-specific targets of oxidation.

When phagocytes are stimulated by interleukin 1, aggregated IgG, complement, or bacterial products such as LPS or fMLP, the multisubunit plasma membrane enzyme NADPH-oxidase is assembled, which produces large amounts of $O_2^{\bullet-}$ directed into the phagosome and extracellular space:

$$O_2 + NADPH \longrightarrow O_2^{\bullet-} + NADP^+ \tag{1.12}$$

This will dismutate to H_2O_2 (spontaneously or *via* SOD) (Equation 1.1). While the toxicity of H_2O_2 and $O_2^{\bullet-}$ is limited, they are used by inflammatory cells as precursors to generate further highly toxic species (Figure 1.7). Neutrophils also release granular enzymes, including myeloperoxidase (MPO) which generates HOCl from H_2O_2 and Cl^-:

$$H_2O_2 + Cl^- + H^+ \longrightarrow HOCl + H_2O \tag{1.13}$$

HOCl is a potent oxidant critical in the cytotoxicity of neutrophils. It reacts readily with a range of biological molecules such as thiols, iron sulfur centres, amino acids, haemoglobin, cytochromes and nucleotides.[87,88] Reaction with amino groups results in the formation of chloramines:

$$RNH_2 + HOCl \longrightarrow RNHCl + H_2O \tag{1.14}$$

In the intestine, ammonia produced by intestinal bacteria can undergo this reaction, generating the potent oxidant monochloramine.[89] Chloramines are longer-lived species than HOCl, and retain the oxidising equivalents of HOCl, thus extending the toxicity of HOCl.[90]

In addition to oxidants, neutrophils release proteases, including collagenase, gelati-

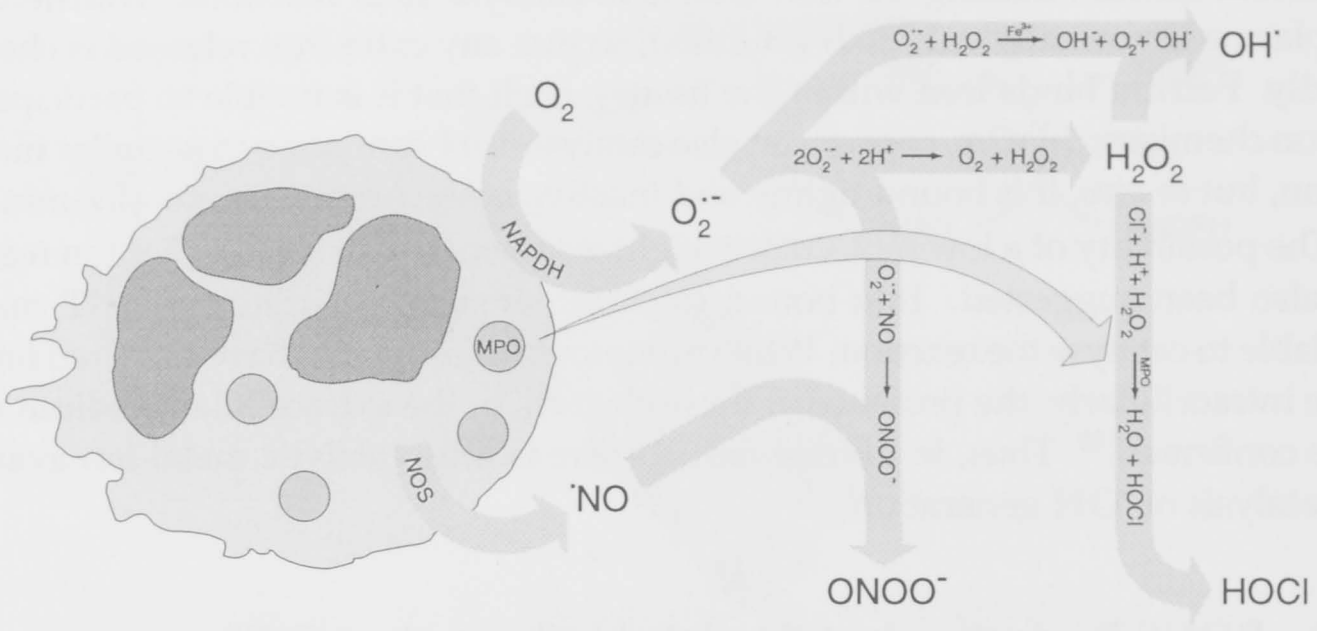


Figure 1.7: Production of reactive oxygen and nitrogen species by neutrophils.

nase, and elastase, for degradation of the extracellular matrix, although collagenase and gelatinase are released in a pro-enzyme form and elastase is balanced by the presence of inhibitors such as α_1-antiproteinase. However, the destructive potential of these enzymes is realised by neutrophil-derived HOCl, which activates the latent collagenase and gelatinase, and inactivates α_1-antiproteinase such that a protease-rich inhibitor-poor zone may be created around the neutrophil, enabling enzymic degradation of the extracellular matrix.[28,91]

Stimulation of inflammatory cells also results in the expression of iNOS for production of $^{\cdot}NO$ (Equation 1.7). $^{\cdot}NO$ is central to the cytostatic and cytotoxic potential of macrophages, which is mediated by the reaction of $^{\cdot}NO$ with iron-sulfur centres of enzymes, including the DNA synthesis enzyme ribonucleotide reductase, and elements of the mitochondrial respiratory chain.[71] The role of $^{\cdot}NO$ in neutrophil cytotoxicity, when MPO is functional, is less clear.[71,92] Neutrophil $^{\cdot}NO$ production also mediates formation of the potent carcinogens, nitrosamines.[93]

The simultaneous production of $^{\cdot}NO$ and $O_2^{\cdot-}$ by activated macrophages and neutrophils can result in the production of a further cytotoxic species, $ONOO^-$:[94,95]

$$^{\cdot}NO + O_2^{\cdot-} \longrightarrow ONOO^- \tag{1.15}$$

While $^{\cdot}NO$ is relatively stable and membrane permeable, $ONOO^-$ is charged and will not diffuse through membranes. While ionised it is quite stable, however when protonated (pK_a of 6.6), peroxynitrous acid decays rapidly to produce the highly toxic $^{\cdot}OH$:[96]

$$ONOO^- \longrightarrow ONOOH \longrightarrow {}^{\cdot}OH + {}^{\cdot}NO_2 \tag{1.16}$$

It has been suggested that this reaction may be an important contributor to the toxicity of $O_2^{\cdot-}$.[96]

$^{\cdot}OH$ can also be generated by the Fenton reaction of H_2O_2 and $O_2^{\cdot-}$ (Equation 1.4). While in normal tissue there is little catalytic metal-ion available for catalysis of $^{\cdot}OH$ generation, in pathological situations iron can become available from several sources. Microbleeding and subsequent degradation of haemoglobin can release haem for $^{\cdot}OH$ generation. Excess $O_2^{\cdot-}$ and H_2O_2 may be involved in this degradation, which can produce the highly reactive ferryl and perferryl radical intermediates.[85,86] Alternatively, iron may be mobilised from transferrin by a decrease in pH,[97] or from ferritin by $O_2^{\cdot-}$[98] or $^{\cdot}NO$,[99] conditions which potentially exist within the phagosome or in the vicinity of activated phagocytes.

1.2.4 Tissue Damage by RONS

Although RONS are a normal part of oxidative metabolism and cells have many mechanisms of protection against them, in situations of disease where elevated production of RONS or depletion of antioxidant defenses may occur, such as inflammation, RONS have the potential to damage many molecules necessary for cellular function.

Many radicals can initiate lipid peroxidation chain reactions which has immediate consequences for membrane integrity and oxidation of membrane proteins.[86] Lipid peroxidaton can be initiated by any radical sufficiently reactive to extract a hydrogen atom from an unsaturated fatty acid, such as $^{\cdot}OH$. The lipid radical is then rearranged for greater stability and can take up molecular oxygen to form the peroxyl radical. Peroxyl radicals can then abstract hydrogen from further fatty acid side chains in a membrane and so propogate the chain reaction of lipid peroxidation. Thus, one initiating event can result in the conversion of vast numbers of fatty acid side-chains to lipid hydroperoxides.[86] Antioxidants which are capable of interrupting this radical propagation process are the chain-breaking antioxidants, and include primarily α-tocopherol in the lipid phase and ascorbate in the aqueous phase (Section 1.2.2.2).

Lipid radicals and intermediates may oxidise membrane proteins, such as calcium channels[84,100] or receptors,[101] with important consequences for cell function. Cytosolic proteins are also susceptible to oxidation, with enzymes containing thiols at the active site most vulnerable to inactivation,[102] including those involved in energy metabolism such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH).[103] Other outcomes of protein oxidation include nitration of tyrosine residues by $ONOO^-$,[104] carbonyl formation,[105] fragmentation and crosslinking[106] and increased susceptibility to degradation by proteases.[107,108]

DNA is also susceptible to oxidative attack by H_2O_2, $^{\cdot}OH$ and $^{\cdot}NO$, which may result in single strand breaks in cellular DNA or base modification, such as the hydroxylation of guanosine by $^{\cdot}OH$.[109–111] These changes may be lethal to cells, or in situations of repeated modifications, may be involved in carcinogenesis.[112,113]

1.2.5 Diseases Involving RONS

There is growing evidence of the involvement of oxidant production and tissue injury processes in many diseases, including ischaemic reperfusion injury, rheumatoid arthritis and athersclerosis. Reperfusion injury occurs to an organ during the resumption of blood flow following a period of ischaemia. As mentioned earlier (Section 1.2.1.1), during ischaemia, limited proteolysis or thiol oxidation converts xanthine dehydrogenase to the $O_2^{\bullet-}$-generating form of the enzyme xanthine oxidase. At the same time, catabolism of ATP leads to increased concentration of the purine metabolites hypoxanthine and xanthine. Reperfusion supplies the other substrate, O_2, and a sudden burst of $O_2^{\bullet-}$ is generated. Tissue damage may result from subsequent $^{\bullet}OH$ generation, or from the attraction and activation of neutrophils by $O_2^{\bullet-}$. The reduction of injury by the antioxidants SOD, catalase, $^{\bullet}OH$ scavengers or the xanthine oxidase inhibitor allopurinol, given prior to reperfusion, demonstrate the importance of these oxidants to the injury process. This process may occur in almost any tissue, although the heart is of particular interest in the context of human disease, and the application of the knowledge of reperfusion injury to transplantation procedures may be important in improving transplant outcomes.[70,114]

The chronic inflammation of rheumatoid arthritis is believed to be accompanied by increased RONS generation in the joint. The synovial fluid from the joints of patients with rheumatoid arthritis is of lower viscosity that normal synovial fluid. An interest in the involvement of oxidants arose when a similar decrease in viscosity was produced *in vitro* by exposing hyaluronate to $O_2^{\bullet-}$. Further work has shown this is due to iron-dependent $^{\bullet}OH$ formation and depolymerisation of hyaluronate. Free iron has been detected in the synovial fluid from inflamed knee joints of rheumatoid arthritis patients, which may become available from cell lysis or microbleeding and haemoglobin degradation, and $O_2^{\bullet-}$ may be derived from activated neutrophils within the joint, macrophage-like cells in the pannus, or possibly from cycles of hypoxia-reperfusion. Thus conditions appear favourable for $^{\bullet}OH$ generation *in vivo*.[85,115] The ability of many non-steroidal anti-inflammatory drugs to inhibit neutrophil production of RONS or to scavenge RONS further supports the involvment of RONS in the joint damage of rheumatoid arthritis.[116–119]

Evidence strongly indicates that oxidation of low density lipoproteins (LDL) is an early event in atherosclerosis. Early atherosclerotic lesions are characterised by an accumulation of lipid-laden macrophages or foam cells in the subendothelial space. An important aspect of the development of these lesions is how these cells take up lipids, which are primarily derived from lipoproteins.[120] Oxidation of LDL converts these molecules into a high-uptake form, although the precise mechanisms of oxidation which may be responsible *in vivo* are not known.[86] Immunocytochemistry has shown the presence of oxidised LDL within the atherosclerotic plaques, and lipophilic antioxidants in animal models of athersclerosis have reduced disease significantly. However, the use of antioxidant or vitamin supplements for prevention of atherosclerosis in humans is yet to be proven, and there are many other contributing risk factors to this disease.[121]

1.3 Reactive Oxygen and Nitrogen Species in Inflammatory Bowel Disease

The active IBD lesion is characterised by a dense inflammatory infiltrate of phagocytes and lymphocytes which, when activated, release a plethora of species which are currently believed to be responsible for the mucosal tissue damage of IBD (Section 1.1.3). Included in this assault is the production of RONS, for which a considerable amount of literature supports a role in IBD. Evidence of increased oxidant production and reduced antioxidant defences in IBD has been reported, and results with antioxidant therapies, including evidence from animal models, further supports a role for RONS in pathogenesis. The interest in RONS and IBD has lead to the formulation of hypotheses of their possible role in initiation of inflammation and in the carcinogenesis associated with chronic UC. However, the understanding of how RONS are involved in the tissue injury and disease process of IBD is limited, as described in the sections below.

1.3.1 Increased RONS Production

Several studies have demonstrated that peripheral blood monocytes and neutrophils from patients with active IBD have a greater ability to produce RONS when stimulated *in vitro*.[122–124] In one report, serial analyses revealed RONS-generating capacity of neutrophils was markedly enhanced with clinical deterioration of the disease, and returned to normal levels with disease improvement.[125] These observations may be of little relevance if were it not for the demonstration by other investigators of the uptake of peripheral blood monocytes and neutrophils into the actively inflamed lesion in IBD.[18–20] Furthermore, macrophages isolated from inflamed mucosa of IBD patients have been shown to have a greater capacity to undergo a respiratory burst when stimulated *in vitro*, compared to those isolated from non-inflamed mucosa.[126]

Within the mucosa, a several-fold increase in MPO levels throughout the colonic mucosa in patients with ulcerative colitis has been reported, representing the potential for oxidant production by neutrophils.[127] Increased generation of RONS by inflamed mucosa has been demonstrated by elevated chemiluminescence of whole biopsies.[128–130] This chemiluminescence was inhibited by the MPO inhibitor azide, the HOCl scavenger taurine and catalase, suggesting that neutrophil-derived oxidants are generated in the mucosa in active IBD.[128,129] Similar experiments have been done in experimental colitis induced by mitomycin-C or acetic acid.[130] Cyclooxygenase and 5-lipoxygenase, whose activity is elevated in IBD mucosa,[25,131] can produce radical intermediates capable of initiating lipid peroxidation, however, inhibitors of these enzymes did not decrease biopsy chemiluminescence, suggesting that these pathways were not major sources of RONS in the inflamed intestine.[129,130]

The recent interest in $^{\cdot}NO$ has lead to the examination of $^{\cdot}NO$ production in IBD. Mucosal $^{\cdot}NO$ production in IBD patients has been measured directly, with 100-fold higher luminal $^{\cdot}NO$ gas concentration being found in ulcerative colitis patients compared to control patients.[132] Indirectly, elevated levels of citrulline[133] and NOS activ-

ity[134] in biopsies of inflamed mucosa were also suggestive of increased $^{\cdot}NO$ generation within the inflamed mucosa. Increased $^{\cdot}NO$ production has also been implicated in experimental colitis by the detection of increased systemic nitrite levels or colonic NOS activity in chronic colitis in rhesus macaques[48] and inflammation induced by TNBS or PG-PS.[57,135,136]

Iron-mediated oxidation is another proposed mechanism through which oxidants may play a role in IBD. The occurrence of hydroxyl radical generation through Fenton chemistry *in vivo* is debatable because of the normally low level of saturation of iron binding proteins in tissues and the question over the availability of iron to catalyse the reaction (Section 1.2.2.3). Babbs[137] proposes that in the inflamed colon, there is abundant iron available to catalyse these reactions and contribute to the tissue damage of colitis. The proposed sources of iron are unabsorbed dietary iron, concentrated in the faecal material, especially in cases of oral iron therapy for chronic anaemia, and mucosal bleeding, characteristic of UC, supplying catalytic haem iron. Thus, Fenton chemistry, driven by phagocyte-generated $O_2^{\cdot-}$ and H_2O_2, may generate $^{\cdot}OH$ with consequent tissue injury.

1.3.2 Depletion of Antioxidants

While oxidant production in the inflamed mucosa appears to be elevated, this may not lead to oxidative tissue injury if the antioxidant capacity of the mucosa is sufficient to remove them. The normal human colonic mucosa has a relatively low enzymic antioxidant capacity, with enzymic antioxidant activities only approximately one tenth that of the liver.[138] Studies of enzymic antioxidants in IBD have only examined SOD activity, which was found to be decreased in granulocytes and inflamed mucosa of patients with UC and CD.[139,140] The chemical antioxidants ascorbate,[141] urate, ubiquinol-10[142] and glutathione[142,143] have also been shown to be depleted in the inflamed mucosa of IBD patients. Not only were total levels of ascorbate depleted, but dehydroascorbate reductase activity was significantly decreased, suggesting that the ability of the mucosa to maintain reduced ascorbate concentrations was diminished.[141] Thus mucosal antioxidant capacity is compromised in IBD, leaving the tissue vulnerable to further oxidative stress.

In experimental colitis two studies have reported on mucosal SOD activity during inflammation, with decreased SOD activity found in TNBS-induced colitis,[144] while an increase in SOD in acute acetic acid-induced colitis was found.[145] Other studies in animals have illustrated the importance of GSH and reduced thiols in intestinal function, with inhibition of GSH synthesis in mice by administration of buthionine sulfoximine (BSO)[146,147] or the intraluminal administration of the thiol blocker, iodoacetamide,[148] resulting in colitis. However, reports on the levels of other enzymic antioxidants or the status of chemical antioxidants in established models of colitis are lacking.

1.3.3 Oxidative Tissue Injury

While considerable evidence supporting increased oxidant production in the inflamed mucosa of IBD exists and the depletion of antioxidants suggests a diminished capacity to cope with oxidant stress, there are very few studies showing that oxidative tissue injury is present in the inflamed mucosa. Direct evidence of oxidative tissue injury in IBD was provided by the finding of oxidative inactivation of the thiol-containing glycolytic enzyme GAPDH. GAPDH activity and its ability to bind the reduced thiol-specific probe, [^{14}C]-iodoacetamide ([^{14}C]-IAM), were consistently reduced in the colonic epithelial cells isolated from inflamed compared to non-inflamed mucosa from IBD patients.[149] Correspondingly, exposure of isolated human colon epithelial cells to oxidants *in vitro* resulted in loss of GAPDH activity and [^{14}C]-IAM binding, suggesting that oxidants may be involved *in vivo*.[149] One other study found higher concentrations of lipid peroxides in rectal biopsies from IBD patients which were normalized concomitantly with a significant improvement in disease activity.[150]

Other evidence is less direct, but supports the possibility that oxidants mediate the injury observed in colitis. The colitis that results from the intraluminal administration of $ONOO^-$[151] or the radical generator, 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH),[152] demonstrates that RONS are capable of inducing the intestinal mucosal injury of colitis. Reactive oxygen species are also suggested to be involved in colitis induced by TNBS, as metabolism of TNBS by rat colon homogenates or isolated colonocytes produces $O_2^{\cdot-}$ and H_2O_2.[52] Increases in the permeability of mucosa *in vivo* or epithelial cell monolayers *in vitro* are observed after oxidant exposure.[153,154] This may be a result of oxidant toxicity to intestinal epithelial cells, which has been demonstrated by several investigators.[153,155,156]

This limited amount of direct evidence of oxidative tissue injury in IBD has lead to hypotheses being put foward on the involvement of oxidant-mediated damage in initiation of inflammation of the intestine, and on the increased incidence of carcinogenesis associated with prolonged IBD. Grisham and Granger have hypothesised that ischaemia reperfusion injury may be involved in initiating inflammatory events in IBD.[157] During transient reduction in colonic blood flow (in response to sympathetic nervous activity as a result of stress) conversion of xanthine dehydrogenase to xanthine oxidase may occur, leading to the formation of RONS in the mucosa upon reperfusion. This burst of RONS may lead to subtle changes in mucosal structure, such as increased permeability of the epithelium or degradation of the mucin coat, which could result in bacterial products penetrating the mucosal barrier. Lamina propria phagocytes may be activated and attracted, producing further chemoattractants and oxidants, and leading to an inflammatory cascade of events. Thus, transient ischaemia could initiate intestinal inflammation *via* free radical production.[157] This scheme is of most relevance to the small intestine where xanthine oxidase is abundant.[158]

Patients suffering from ulcerative colitis for many years are at increased risk of developing colon cancer. A link between oxidant production and cancer in colitis has been suggested by several investigators. Babbs, who suggested iron-mediated oxidation may be important in the tissue damage of IBD (Section 1.3.1), speculated

further that $^{\cdot}OH$ and other products of luminal Fenton chemistry may be involved in carcinogenesis and tumour promotion in the mucosa, especially in patients suffering from chronic UC.[137,159] Excessive $^{\cdot}NO$ production, as would occur during chronic gut inflammation, also has the potential to promote carcinogenesis *via* the formation of the highly carcinogenic nitrosamines.[160,161]

1.3.4 Antioxidant Therapies in IBD

RONS are further implicated by the efficacy of IBD treatments which have antioxidant properties. The possible mechanisms by which 5-ASA may be effective in IBD are many,[33] including numerous antioxidant actions. 5-ASA and sulfasalazine are potent scavengers of RONS, including hypochlorite, hydroxyl, superoxide and peroxyl radicals.[116,162–164] Oxidant production may also be inhibited, as aminosalicylates inhibit MPO,[119] neutrophil chemiluminescence,[162] and also the chemiluminescence of rectal biopsies from patients with active disease.[128] Tissue injury may be prevented, as 5-ASA inhibits lipid peroxidation *in vitro* by iron/ascorbate,[165] can protect α_1-antiproteinase from inactivation by HOCl,[163,166] and protects cultured cells from damage by H_2O_2 and $O_2^{\cdot-}$.[167]

While these studies examined 5-ASA *in vitro*, an antioxidant mechanism of action for 5-ASA *in vivo* in IBD has been suggested by the finding of oxidised metabolites of 5-ASA in the faecal material of IBD patients.[150] Further, direct use of the antioxidant enzyme, SOD, has been trialled in IBD. In a phase II trial of intramuscular injections of SOD on 26 CD patients, very positive outcomes in the short and long term were observed in 73–82% of patients.[39] The success of this study encourages further examination of antioxidant therapeutic strategies.

Indeed, many other antioxidant strategies have been examined in animal models of colitis with encouraging results. In acetic acid-induced colitis in rats, scavengers of $O_2^{\cdot-}$ were found to decrease the inflammatory score of animals, while agents intended to decrease $^{\cdot}OH$ production or xanthine oxidase inhibitors did not.[168] A further study on this model found the anti-RONS agents catalase, WR-2721, a thiol compound that is a GSH-sparing agent, and copper (II) 3,5-diisoproylsalicylic acid, a SOD mimic, also significantly decreased the severity of colonic inflammation and mucosal chemiluminescence, indicating that oxidants, in particular $O_2^{\cdot-}$ or H_2O_2, may play an important role in acetic acid-induced colitis.[169] In fMLP-induced colitis, manganese-loaded desferrioxamine (a SOD mimetic), Ebselen (a glutathione peroxidase analogue), desferrioxamine (an iron chelator), or dimethylsulfoxide (a $^{\cdot}OH$ scavenger) significantly attenuated fMLP-induced increases in mucosal permeability.[170]

The use of NOS inhibitors in models of colitis has implied a role for $^{\cdot}NO$ in the pathogenesis of disease. Inhibition of $^{\cdot}NO$ generation ameliorated increases in macroscopic colonic inflammation scores and colonic MPO content in TNBS ileitis and PG-PS granulomatous colitis.[135,171] A further study in TNBS ileitis using immunohistochemistry revealed that the inhibition of NOS was associated with a decrease in nitrotyrosine staining of the tissue, and thus protein nitration may be involved in the pathogenesis of disease.[172]

While these studies demonstrate the potential for antioxidant therapies to be clinically useful in IBD, these studies, with the exception of the nitrotyrosine study, have thus far looked only at general indicators of inflammation for assessment of treatment outcome. Our understanding of the mechanism of action of these treatments is still limited.

1.4 Aim

While there is considerable indirect evidence suggesting the involvement of RONS in the pathogenesis of IBD, little work has been done on identifying the oxidative modifications which may be occurring in the inflamed tissue. Thus, my aim is to investigate the molecular nature of the oxidative tissue injury in DSS-induced colitis. This will include exploring the similarities of this model to the human disease in terms of what is already known about the oxidative changes in the mucosa from IBD, and looking for new evidence of oxidative injury in DSS-induced colitis with a view to gaining a better understanding of the human disease process and examining the possibility of antioxidant therapies.

To this end, Chapter 3 examines the MPO activity and $^{\cdot}OH$ generation as indicators of the possible oxidant production occurring in the inflamed and normal mucosa. Chapter 4 explores the status of the non-enzymic antioxidants and thiol content in the normal and inflamed mucosa. Total scavenging capacity of the mucosa was assessed, and ascorbate, ubiquinol, α-tocopherol and urate levels within the mucosa were measured. The reduced thiol content of the mucosa was determined, and the GAPDH activity and [^{14}C]-IAM-binding assessed as indicators of thiol and protein thiol oxidation. The status of these molecules in the inflamed human intestine has been characterised previously, allowing an assessment of the similarity of the tissue changes in DSS-induced colitis to those of IBD.

In Chapters 5 and 6, evidence of oxidative modification to mucosal proteins was sought. By employing the technique of western blotting to the assessment of protein carbonyls and nitrotyrosine, identification of specific proteins which had been modifed was possible. These investigations were performed on both the model colitis and on IBD tissue to determine whether these forms of oxidant injury may be involved in the mucosal damage of colitis.

Chapter 7 draws these studies together, and assesses the value of the various approaches available for the investigation of oxidative tissue injury in IBD, with discussion of how studies of this nature contribute to the understanding of the disease process of IBD, and hence to the rational development of therapies.

Chapter 2

Dextran Sulfate-Induced Colitis

2.1 Introduction

Many animal models of colitis are available (Section 1.1.5), however, the chronic and relasping nature of the human disease have been particularly difficult to mimic.[43] DSS-induced colitis in mice was characterised in an attempt to provide a reliable chronic model, and has been described as both an acute or chronic form of disease in two recent reports.[60,61]

Sulfated polysaccharides have been known to induce intestinal inflammation, with oral administration of low molecular weight carrageenan used previously to induce inflammation of the caecum and colon. Carrageenan is isolated from seaweed, and difficulties with this model arose from variability in its composition. DSS is a synthetic, high molecular weight (30-40 kDa), highly sulfated polysaccharide, which, like carrageenan, when administered orally induces colitis. The synthetic origin of the polymer, and hence consistency in the makeup, makes its use preferable to carrageenan.

Oral administration of DSS has induced both acute and chronic colitis in many strains of mice, and also rats and guinea pigs. Acute colitis in mice is induced by the administration of 5% DSS in drinking water for 7–11 days. The disease is characterised by weight loss and the appearance of diarrhoea and rectal bleeding. Macroscopically it bears similarities to ulcerative colitis, with inflammation which is confined to the colon, primarily affecting the descending and transverse regions, and to Crohn's disease, with patchy rather than continuous inflammation. Shortening of the colon is observed, with thickening of the muscle layer. Histologically, inflammatory cell infiltration, including polymorphonuclear leucocytes, multiple erosive lesions, and focal disappearance of mucosal crypts were observed in the colonic mucosa.[60] Inflammation was mucosal, granulomas were absent, and little cryptitis or crypt abscess formation was evident.[61] Immunohistochemically, neutrophils have been detected in the colon on day 4 with subsequent arrival of inflammatory macrophages, detected on day 6.[173] While the severity of disease varies between strains, within a strain the disease shows great uniformity between animals.

Chronic colitis can be induced by 7 days of oral 5% DSS followed by 14–21 days of water or by giving 2–3 cycles of 7 days of DSS, 7–10 days water.[60,61] Histological features of this chronic colitis include areas of erosions and inflammation with

areas of inactivity, crypt distortion, florid epithelial proliferation and possible dysplasia.[61] This characteristic may be useful in studying the colitis-dysplasia-cancer sequence.[43,61,174,175] While a very useful chronic model, it lacks the relapses of the human disease.

The mechanism of induction of colitis in this model is not known. Very early histological changes show a shortening of the crypts preceding inflammation, suggesting that inflammation may be secondary to an initial insult at the level of the colonic enterocyte.[61] DSS has been shown to inhibit the proliferation of colonic epithelial cell lines *in vitro* at concentrations likely to be achieved within the colon.[62,176]

DSS colitis does not require the presence of either T cells or B cells for induction of colitis, as it can be induced in nude mice, lacking T cells,[177] and SCID mice which lack functional T cells and B cells, but do have an intact innate immune system, including macrophages, natural killer cells, and granulocytes.[62] Studies in interleukin 5 knockout mice have suggested that eosinophils are not essential for the disease process.[178] Macrophages may be important as DSS is chemotactic to macrophages and mucosal macrophages are found with enlarged lysosomes containing DSS.[60] Depletion of neutrophils results in dramatic reduction in the severity of colitis induced by DSS[179] indicating the importance of neutrophils in the inflammatory process of this disease.

A role for intestinal flora too is possible, as metronidazole has been reported to ameliorate DSS-induced colitis, but the reason for this is unclear.[62,180] Other studies have suggested that intestinal bacteria are not essential for development of disease, as colitis develops in C3H/HeJ mice,[181] a strain which are insensitive to LPS, and in germ-free mice.[182]

Studies on the biochemical mediators of inflammation have shown a 5-fold increase in colonic eicosanoid levels after 7 days of DSS administration to mice, which was prevented by treatment with olsalazine.[183] Elevated mucosal levels of the macrophage-derived cytokines interleukin 1β, interleukin 6 and tumour necrosis factor in the inflamed colons have been reported[62] and interleukin 1-receptor antagonist and transforming growth factor β2 have shown beneficial effects.[43]

Both the chronic and acute forms of disease have been shown to respond to the current therapeutic strategies used in humans, sulfasalazine and olsalazine therapy.[184] Cyclosporin enemas in DSS mice have also been effective.[185] There is no report of beneficial corticosteroid therapy in DSS-induced colitis, and work in our laboratory suggests it is not responsive to sub-cutaneous or oral prednisolone (unpublished data).

While some aspects of DSS-induced colitis are yet to be thoroughly characterised, it has several advantages over other models including the simplicity of administration, a predictable time course and consistency of disease within a given strain. The ability to induce either acute or chronic disease is a feature not found in most other models.[62] This model is appropriate for studying contributions from the innate immune system and non-specific inflammatory processes involved in intestinal inflammation,[43] and its convenience and reproducibility lend it to therapy screening studies. Thus DSS-induced colitis is potentially a highly suitable model for the study of oxidative processes mediated by phagocytes, the role of such processes in the pathogenesis

of colitis, and antioxidant therapeutic strategies. This chapter describes the methodology and main characteristics of DSS-induced colitis as used in this thesis for studies on the role of oxidant-mediated injury in the mucosa.

2.2 Methods

2.2.1 Induction of Colitis

Studies were performed on male CBA/H mice, fed standard mice chow pellets and with *ad libitum* access to drinking water. Over the course of these studies, mice used were aged between 5 and 7 weeks, weighing 18–23 g, with an age range of 7 days and weight range of 2–3 g for any one experiment. Animals were bred in SPF facilities by the ANU Animal Services Division, and after weaning kept in a standard laboratory animal environment. Experiments were performed in accordance with the JCSMR animal ethics guidelines.

Acute colitis was induced by administering distilled water supplemented with 5% DSS w/v (average mol wt 44,000, 15% sulfur content (TdB Consultancy AB, Uppsala, Sweden)) *ad libitum* for 7–11 days.[60] The fluid intake per cage was monitored daily and dextran solutions were changed daily to avoid turbidity. Mouse body weights were recorded daily to monitor weight loss, and the presence of diarrhoea and rectal bleeding was noted. The severity of the symptoms was assessed (slight — soft, unformed pellet, blood on pellet; severe — wet anus, blood on fur around anus) but only the absence or presence of symptoms was plotted.

A less severe colitis was also induced by administering 3% DSS in the same manner for 15 days. The disease course and histology of this moderate colitis were compared to that induced by 5% DSS.

2.2.2 Collection of Mucosa

Mice were sacrificed on the last day of DSS administration and colons dissected. Faecal matter was removed by flushing with phosphate-buffered saline (PBS) and a small section of distal colon was fixed in 10% neutral formalin for standard histological analysis using haematoxylin and eosin staining of 4 μm sections. Histological processing and staining was performed by the Histology and Electron Microscopy Unit of the JCSMR.

The descending and transverse colon was opened longitudinally and the mucosa collected by scraping the tissue with a microscope slide. Mucosa was frozen on dry ice for storage at -70°C and later homogenised in PBS or Hank's balanced salt solution (HBSS) (50 mg tissue/mL) with 10–15 passes of a teflon/glass homogeniser (Wheaton). Aliquots of the homogenates were prepared with various additions for each of the assays and stored at -70°C. The nature of the collected mucosal sample was verified by histology as performed above.

2.2.3 DNA and Protein Content of Mucosal Homogenates

Mucosal homogenates were analysed for DNA and protein content to determine an appropriate measure for standardising other analyses. Protein content of homogenates was determined using the BioRad micro protein assay, based on the Bradford method. Homogenate was diluted 100–200-fold with PBS and centrifuged at 15,000×g 5 min. 750 μl of sample was then mixed with 200 μl of BioRad protein dye concentrate and the absorbance at 595 nm was read 10–30 min later (Varian Cary 1 UV/visible spectrophotometer). Protein concentration was determined against a standard curve of bovine serum albumin of 1–25 μg/ml.

The DNA content of homogenates was determined by fluorescence using the Hoechst 33258 stain according to Brunk *et al.*[186] To aliquots of whole homogenate, an equal volume of assay buffer containing 50 mM sodium phosphate, 2 M NaCl and 2 mM EDTA was added. Homogenates were then put through three cycles of rapid freezing and thawing, and sonicated to release DNA from the nuclei. Samples were centrifuged and 20–40 μl of supernatant was added to a cuvette containing 2 ml of 2 μg/ml Hoechst 33258 in assay buffer. The DNA content was determined by comparing the increase in fluorescence (Ex 356 nm, Em 460 nm, Hitachi F-3000 fluorospectrophotometer) due to addition of supernatant to the increase produced by addition of 0.5–5 μg calf thymus DNA.

2.3 Results

Mice exposed to 5% DSS developed symptoms of acute colitis, with diarrhoea being observed first followed by rectal bleeding, 1–2 days later. Weight loss in these mice during the last few days of exposure was dramatic. In contrast, administration of 3% DSS induced a more moderate colitis, with diarrhoea developing gradually over the 15 days, and only a small amount of rectal bleeding being observed. Unlike their control litter mates, these mice did not gain weight (Figure 2.1).

Histologically, in the moderate colitis, there were a small number of lesions, with loss of crypts in small areas, a mild inflammatory infiltrate, and the surface epithelium appeared intact. In the severe colitis, there were large areas of crypt loss, with a predominantly neutrophilic infiltrate throughout the mucosa. In addition, the surface epithelium was lost, and ulceration and mucosal bleeding were present (Figure 2.2). While the descending and transverse colon were the most severely affected, disease was patchy such that areas of uninvolved mucosa were present around lesions (Figure 2.3).

The mucosa collected from normal and inflamed colons by scraping with a microscope slide was shown by histology to consist predominantly of epithelium and lamina propria, with minimal contamination by muscularis mucosa and submucosal tissue (Figure 2.4). Typical yields of mucosa per mouse (transverse and descending colon) were: control 80–100 mg; inflamed 50–75 mg. The lower yield from the inflamed colons is due largely to the shortening of the colon, and partially to loss of the epithelium.

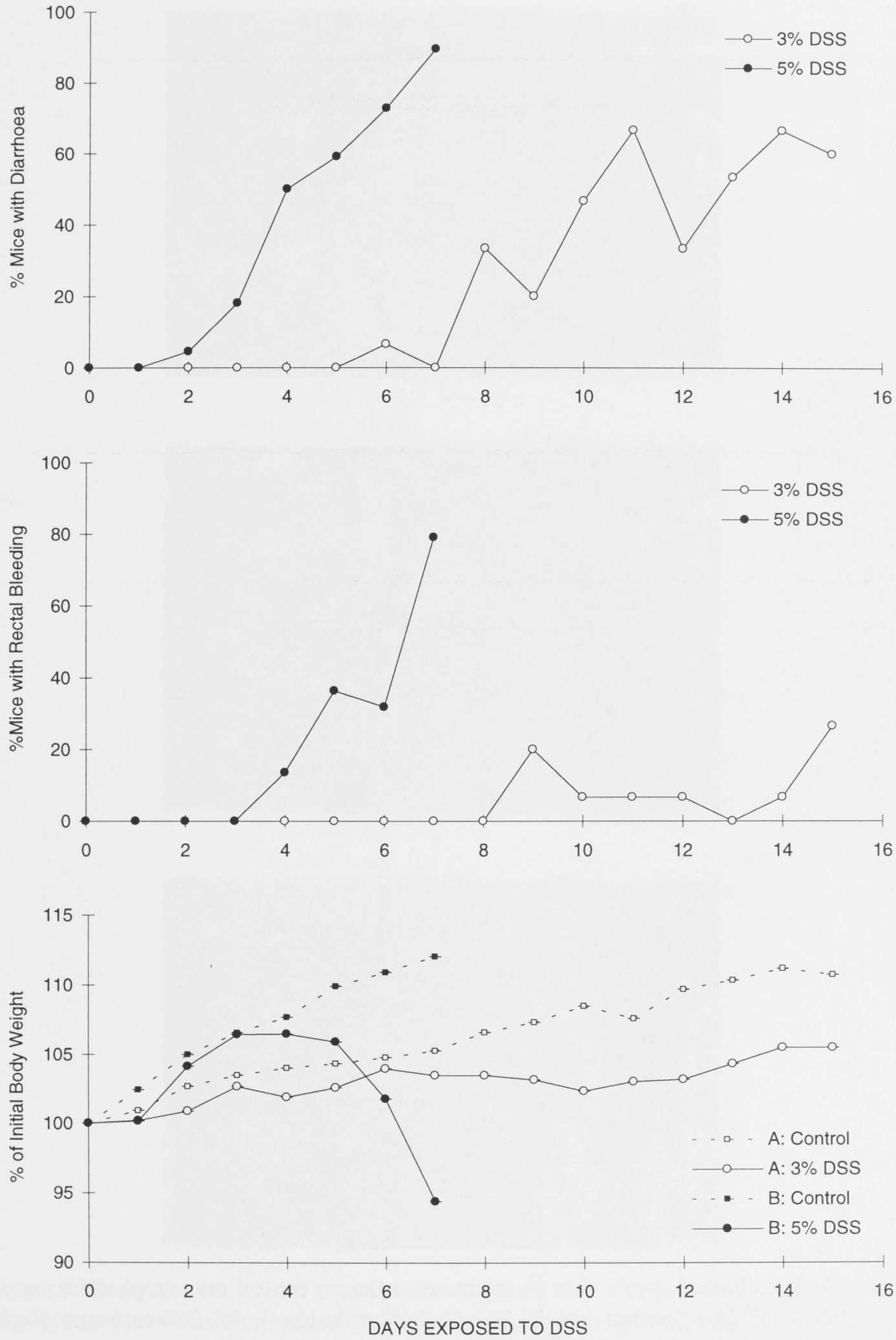
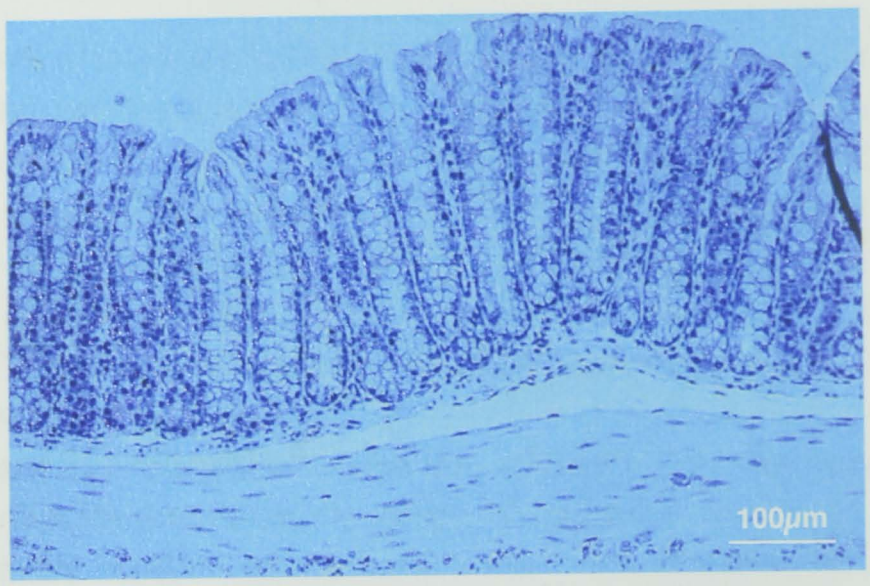
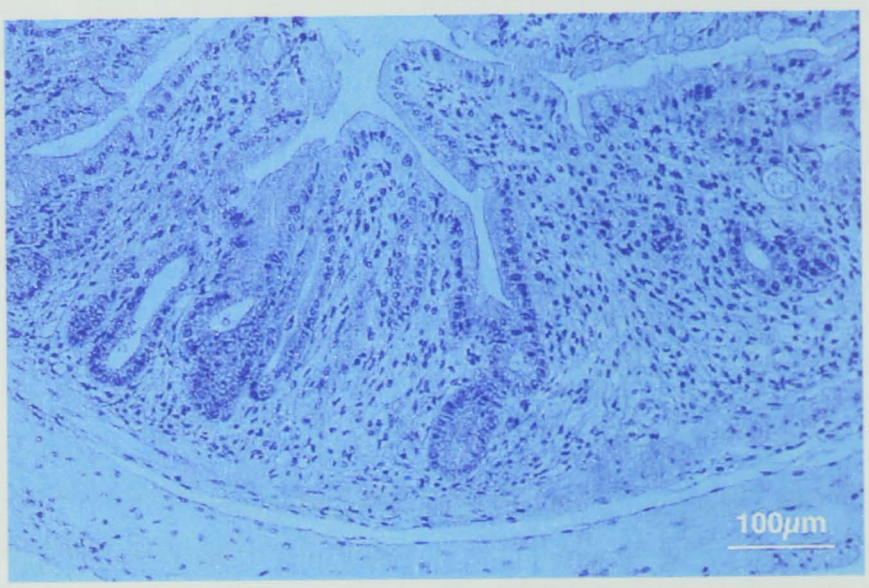


Figure 2.1: Disease course for mice exposed to 3% DSS for 15 days (n=15) and 5% DSS for 7 days (n=22), showing percentage of mice observed with diarrhoea (top) and rectal bleeding (centre). Body weight has been expressed as % of initial weight (bottom).





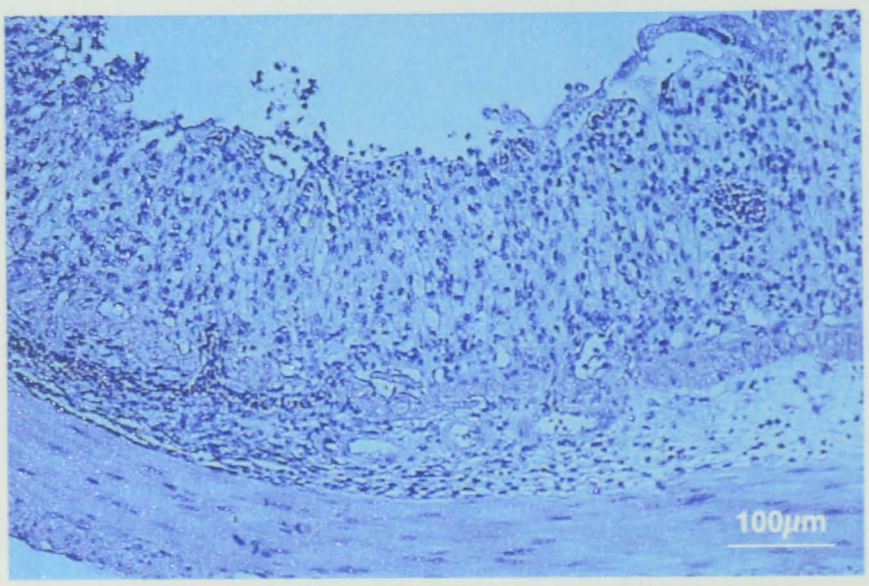
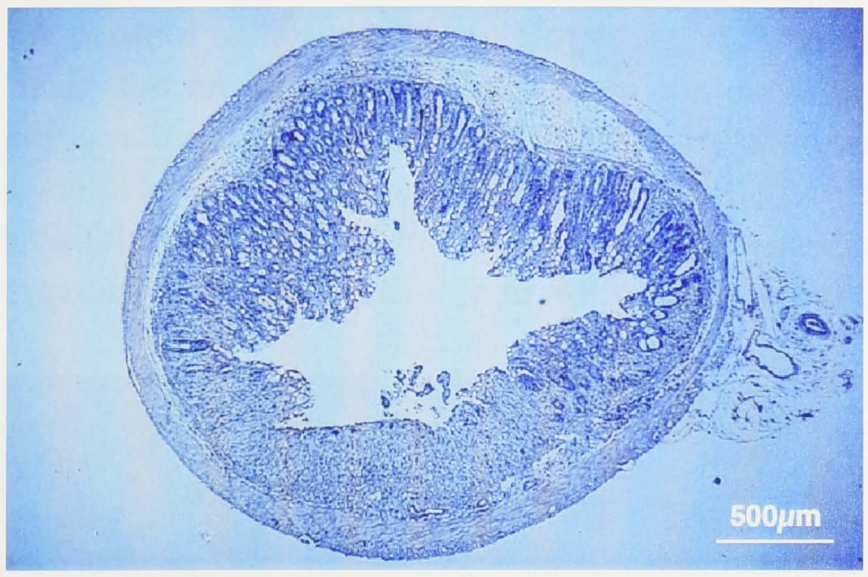
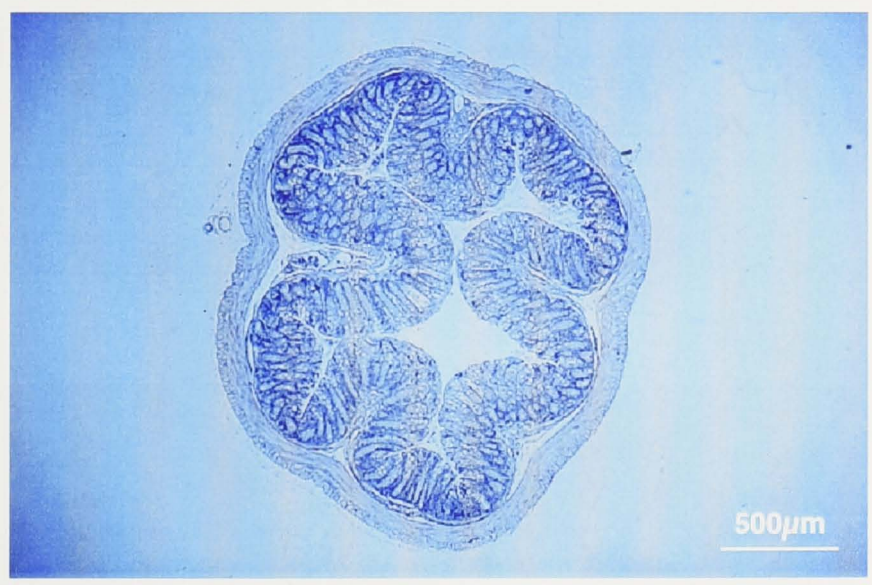


Figure 2.2: Histological appearance of the mouse colon in control mice (top), mice exposed to 3% DSS for 15 days (centre) and 5% DSS for 8 days (bottom). 3% DSS exposure displays loss of crypts without loss of surface epithelium, while 5% DSS exposure results in marked inflammatory cell infiltrate, loss of crypts, ulceration and mucosal bleeding.





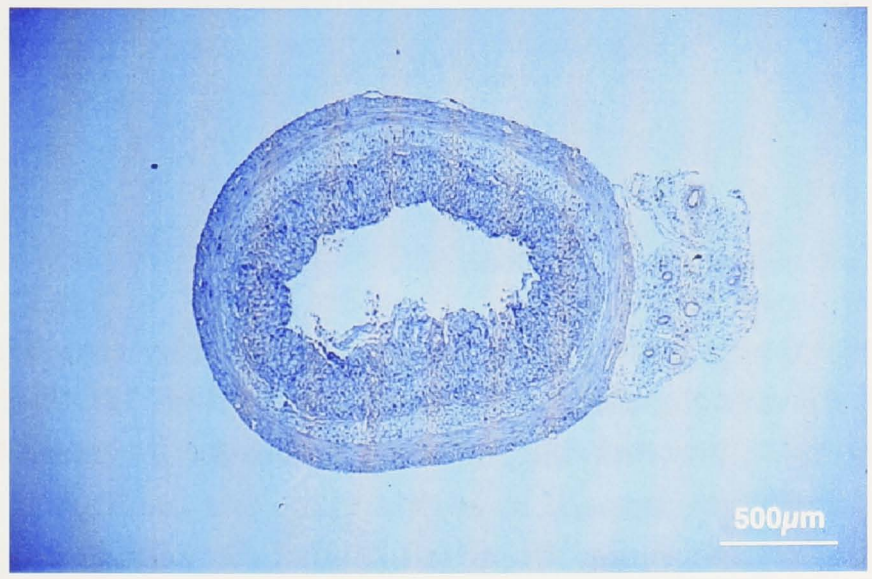
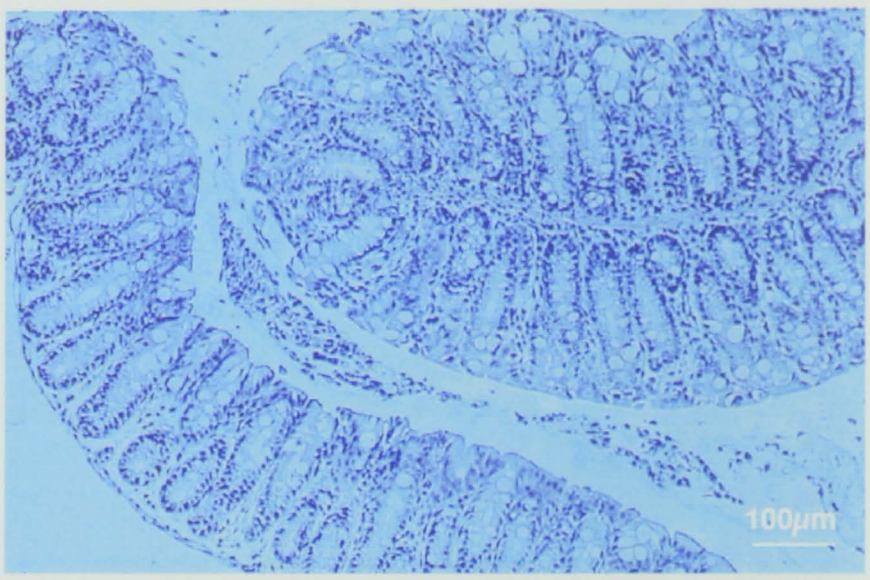


Figure 2.3: Complete sections of mouse colon showing the variability in the distribution of colitis within the descending colon. Colon from a control mouse (top) and mice exposed to 5% DSS for 8 days (centre and bottom) demonstrating the possibility of severely inflamed mucosa occurring adjacent to largely unaffected mucosa (centre), or complete involvement in other areas (bottom).



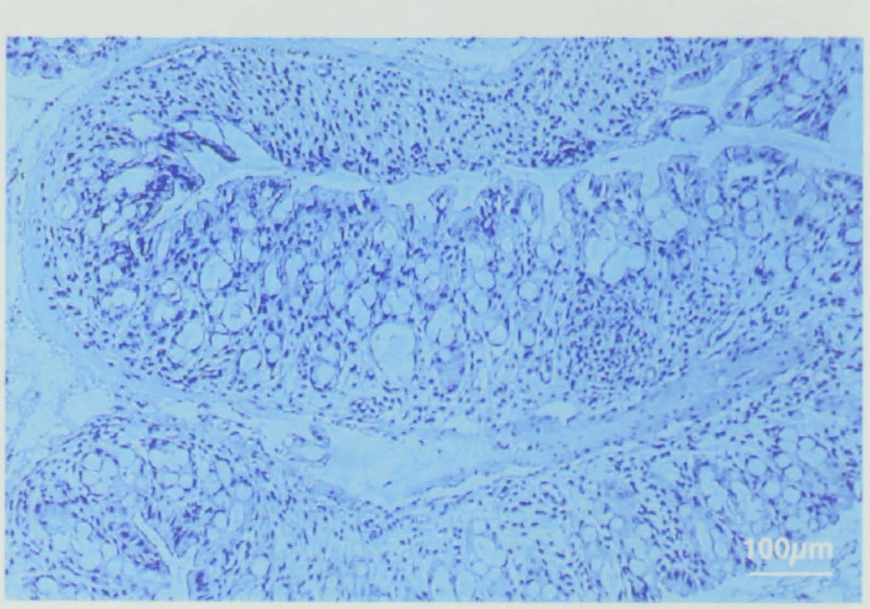


Figure 2.4: Histological appearance of the mucosa collected by scraping with a microscope slide from a normal mouse colon (top) and a mouse exposed to 5% DSS (bottom). Intact crypt structures and lamina propria are clearly visible in the sample from normal tissue.

Protein and DNA analysis of the mucosal homogenates indicated little difference in protein and DNA content of the homogenates prepared from inflamed or normal mucosa (Figure 2.5). In some experiments, the protein content was lower in inflamed than in control homogenates, but this was accompanied by changes in the DNA content also, such that the protein:DNA ratio was the same in the two tissue types. These differences were probably because the inflamed colons are more difficult to clean and dry compared to the normal colons, so that their weights were overestimated.

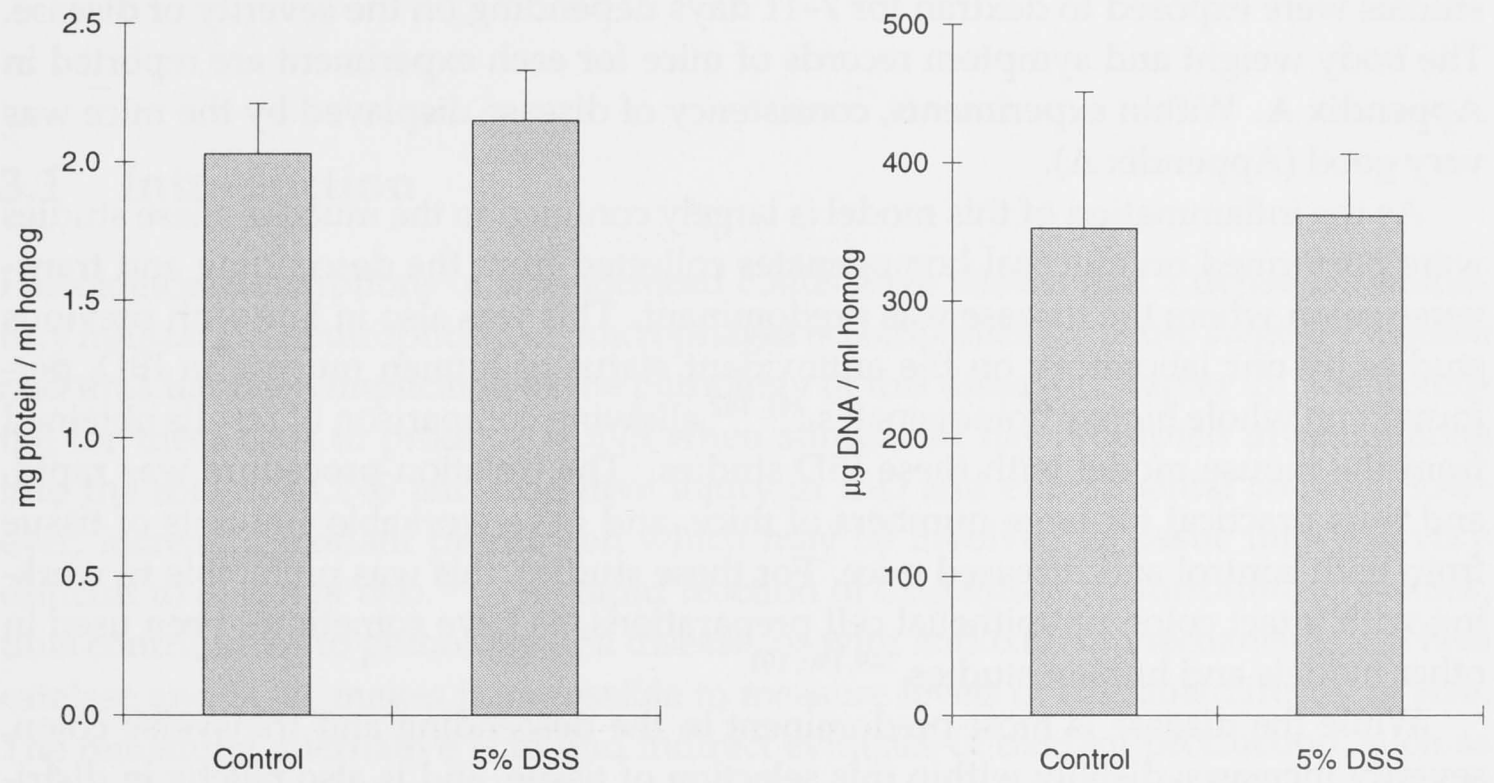


Figure 2.5: Protein and DNA content of mucosal homogenates from control mice and mice exposed to 5% DSS.

2.4 Discussion

The disease induced by administration of 5% DSS in our laboratory was characterised in full when established by Stevceva *et al.*[187] and was found to be very similar to that described by Okayasu *et al.*[60] and Cooper *et al.*[61] The histological features and clinical features presented here are in line with these previous reports on this model.

The 3% DSS-induced colitis described here contrasts the 5% DSS-colitis in the severity of symptoms and the histologic changes observed. The histologic changes in 3% DSS colitis resembled the early stages of disease reported by Cooper *et al.* of epithelial cell degradation, with little inflammation present.[61] The diarrhoea in this disease may result largely from direct toxicity of dextran to epithelial cells[188] rather than inflammatory mechanisms. Secretion may also be promoted by a small increase in oxidant production due to a mild inflammatory infiltrate.[189]

The studies in this thesis aimed to find evidence of oxidative tissue injury in this experimental colitis. For this reason, studies were performed on mice exposed to 5% DSS for 7–11 days, where an intense inflammatory infiltrate, and therefore a large

potential for oxidant production, was observed. The extent of neutrophil infiltration in colitis was confirmed biochemically in Chapter 3 by measuring the MPO levels in the mucosa.

Some variability in the length of exposure necessary to induce this disease was observed over the duration of this project. The major factor was considered to be variations in the litters of mice. It is also possible that exposure of the mice to mouse hepatitis virus, or ectoparasites such as pin worm, increased the sensitivity of the mice to DSS exposure, shortening the timecouse of disease. So mice used in these studies were exposed to dextran for 7–11 days depending on the severity of disease. The body weight and symptom records of mice for each experiment are reported in Appendix A. Within experiments, consistency of disease displayed by the mice was very good (Appendix A).

As the inflammation of this model is largely confined to the mucosa, these studies were performed on mucosal homogenates collected from the descending and transverse colon where the disease was predominant. This was also in line with previous studies by our laboratory on the antioxidant status of human mucosa in IBD, performed on whole biopsy homogenates,[141,142] allowing comparison of results obtained from the mouse model with these IBD studies. The isolation procedure was rapid, and thus practical for large numbers of mice, and gave workable amounts of tissue from both control and diseased mice. For these studies, this was preferable to working with intact colon or epithelial cell preparations, as have sometimes been used in other models and human studies.[149,190,191]

While the disease is most predominent in the descending and transverse colon, severity increases distally within this selection of tissue, and is also patchy in distribution (Figure 2.3).[60,61] The biochemical results reported in this thesis represent the average condition of the descending and transverse colon. Any differences observed between control and diseased mice are likely to be much more severe if it were possible to examine conditions within inflammatory foci. In future studies, division of the colon into smaller segments, for example proximal, middle and distal thirds, may be considered to obtain samples of severly to mildly affected tissue.

The biochemical results presented in these studies have been standardised to the protein content of the mucosal homogenates. The nature of any differences between the inflamed and control tissue reported in this thesis was not altered by standardising the results to DNA or tissue wet weight rather than protein content of the tissue, except in the unusual results reported in Chapter 5. Protein rather than DNA or tissue wet weight was chosen because of the smaller tissue requirement than the DNA assay, and the difficulty in thoroughly drying the tissue for accurate weighing after the removal of faecal material.

While several aspects of DSS-induced colitis have been studied, the biochemical changes in the diseased tissue of this model have not been defined. The presence of infiltrated phagocytes in the inflamed mucosa during this disease indicates the potential for oxidant-mediated injury to be involved in the pathogenesis of this disease. This thesis endeavours to provide evidence of a role for oxidant-mediated tissue injury in the colonic mucosa of mice with acute DSS-induced colitis.

Chapter 3

Oxidant Production in the Mucosa

3.1 Introduction

Histological descriptions of DSS-induced colitis have shown that a dense inflammatory infiltrate of neutrophils and macrophages accompanies the acute stages of disease and thus has been implicated in the pathology of this disease (Chapter 2). The potential for these cells to produce RONS when stimulated has prompted investigations into the role of RONS and oxidative injury in IBD and experimental colitis. However, increased oxidant production which may be involved in tissue injury is very difficult to detect *in vivo*.[115] The rapid reaction of oxidants with surrounding tissues, thus contributing to pathology of a disease, or with antioxidant mechanisms, such as catalase and SOD, makes it impossible to measure levels of oxidants directly *in vivo*. The remaining alternative is to find indirect evidence of oxidant production, such as evidence of increased oxidant sources, or to search for evidence that the oxidant of interest has reacted with another molecule, endogenous or exogenous.

Neutrophils are a potential source of oxidants in DSS-induced colitis. Histology has shown the presence of large numbers of neutrophils in the inflamed lesions, which have the ability to produce a range of oxidants, as described in Section 1.2.3. Particular to neutrophils is the granular enzyme, myeloperoxidase (MPO), which produces HOCl from H_2O_2 and Cl (Equation 1.13). HOCl is one of the most important oxidants that neutrophils produce,[28] and in contrast to H_2O_2 and $O_2^{\bullet-}$, tissues are without a specific removal mechanism for HOCl. MPO activity within the tissue can be determined, and this has been used as an accurate indicator of the extent of infiltration of neutrophils into inflamed tissues in many situations.[191,192]

Hydroxyl radicals ($^{\bullet}OH$) are also highly toxic species, whose rate of reaction is diffusion limited. This radical can be generated from biological oxidants *in vitro via* several pathways (Section 1.2.3) including from Fenton chemistry (Equation 1.4) and $ONOO^-$ decomposition (Equation 1.16). In the inflamed colon, it has been proposed that Fenton chemistry could play a role in tissue injury as there is potentially abundant iron available to catalyse $^{\bullet}OH$ formation, with unabsorbed dietary iron concentrated in the faecal material, and mucosal bleeding supplying catalytic haem iron. Thus, Fenton chemistry, driven by phagocyte-generated $O_2^{\bullet-}$ and H_2O_2, may generate $^{\bullet}OH$ with consequent tissue injury (Section 1.3.1).[137]

While $^{\bullet}OH$ cannot be measured directly, evidence of $^{\bullet}OH$ formation can be ob-

tained by using ·OH traps or detector molecules which form specific products after reaction with ·OH. Salicylate is a ·OH trap which reacts with ·OH to produce the hydroxylation products 2,3- and 2,5-dihydroxybenzoate (DHB), and also in much smaller amounts the decarboxylation product catechol (Figure 3.1).[193] The DHB meta-

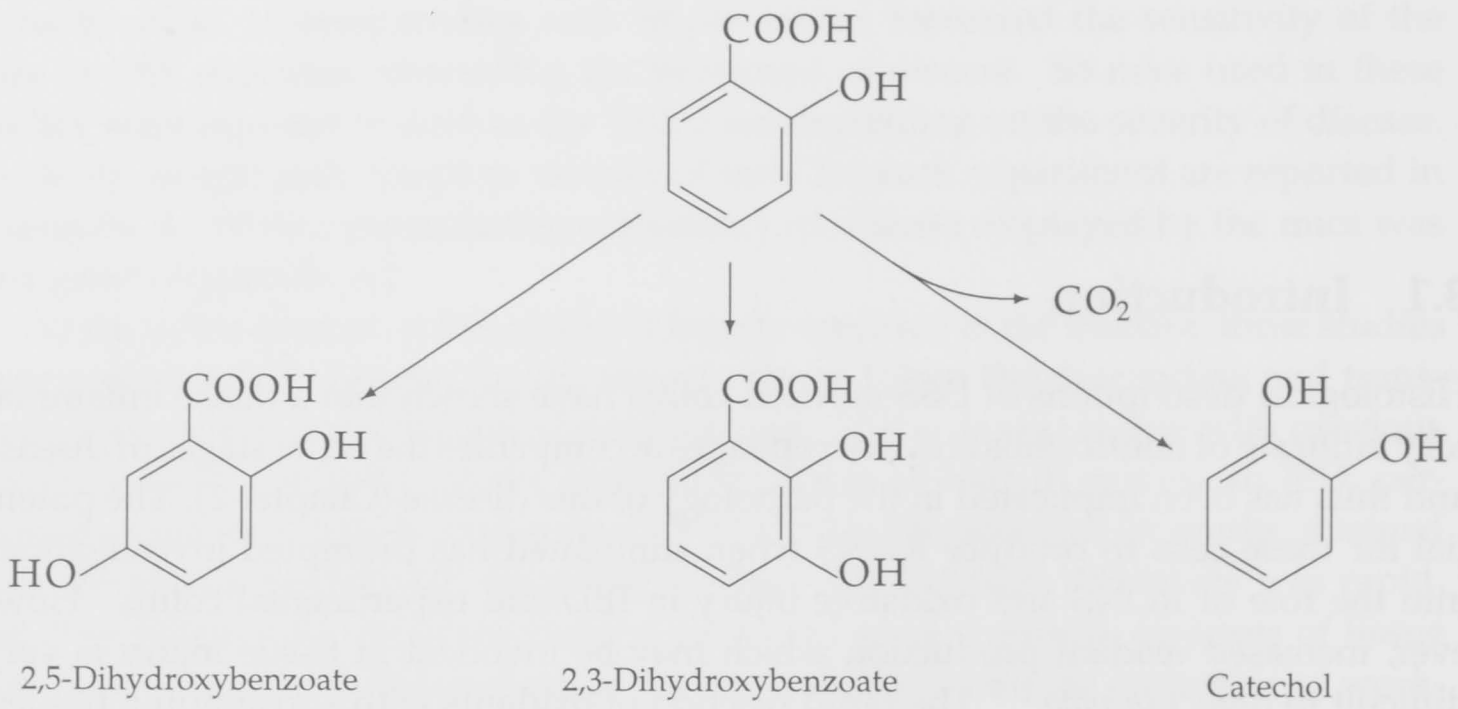


Figure 3.1: Products of the reaction of salicylate with ·OH *in vitro*.[193]

bolites can be separated by high performance liquid chromatography (HPLC) and quantified with electrochemical (EC) detection.[194] The lack of toxicity of salicylate, ease of administration, and stability of the reaction products make salicylate a useful ·OH trap for animal studies. *In vivo*, a wide range of metabolites are produced enzymically from salicylate, including conjugates of glucose and uric acid.[195,196] 2,5-DHB is also produced by the microsomal cytochrome P-450 system[197] and by MPO.[198] However, no enzymic pathway of generation of 2,3-DHB has been identified, thus the formation of 2,3-DHB is considered indicative of ·OH generation *in vivo*.[193,195,197]

Salicylate hydroxylation has been used to measure ·OH in many situations including in the perfusate of isolated organs such as ischaemic/reperfused hearts,[199,200] in intestinal ischaemia in rats,[201,202] and in acute lung inflammation in the rat following instillation of silica.[203] Although the possible involvement of ·OH in colitis has been hypothesised,[137] and ·OH scavengers have been used in models of colitis,[168,170] no attempt has been made to measure ·OH production *in vivo* in colitis.

In this chapter, the possibility of excessive oxidant production occurring in the inflamed mucosa in DSS-induced colitis, which may mediate the tissue injury seen in this disease, was investigated. Specifically, mucosal MPO activity was measured, indicating the potential for neutrophil-mediated oxidation particularly by HOCl, and salicylate hydroxylation was used as an *in vivo* indicator of ·OH production.

3.2 Methods

3.2.1 Induction of Colitis

Colitis was induced in 6–7 week old male CBA/H mice by administering distilled water supplemented with 5% DSS *ad libitum* for 7 days or 3% DSS for 15 days (Chapter 2). The mice used in this study were those described in detail in Chapter 2, thus the disease course for these animals and representative histology can be seen in Figure 2.1 and Figure 2.2 respectively.

3.2.2 Myeloperoxidase Activity

Hexadecyltrimethylammonium bromide (Sigma) was added to homogenates to a final concentration of 0.5%. Homogenates were then sonicated, frozen and thawed three times, sonciated again and microfuged at 15,000×g for 10 min, 4°C. The resulting supernatant was assayed for MPO activity according to Krawisz.[191] A 30 μl aliquot of supernatant was added to a 1 ml cuvette containing 50 mM KH_2PO_4 pH 6.0, 0.0005% H_2O_2 and 0.167 mg/ml *o*-dianisidine (Sigma). The initial rate of reaction was monitored at A_{460}, 25°C (Varian Cary 1 UV/visible spectrophotometer) and the MPO activity of the tissue calculated using an extinction coefficient of 1.13×10^4 $M^{-1}cm^{-1}$ for oxidised *o*-dianisidine.[204]

3.2.3 Measurement of Salicylate Hydroxylation

Sodium salicylate, 2,3- and 2,5-DHB were obtained from Aldrich. Control mice and mice exposed to DSS were injected with sodium salicylate 100 mg/kg i.p. 60 min before sacrifice, and colonic mucosa was collected and frozen on dry ice. Mucosal homogenates were prepared, and trichloroacetic acid (TCA) added to precipitate protein. The TCA supernatant was then analysed for salicylate and its hydroxylation products by HPLC with fluorescence and EC detection.

To examine if the ratio of 2,3-DHB to salicylate varied with dose or time, control mice received 50, 100 or 200 mg/kg salicylate i.p. and were sacrificed 60 min after injection. In time course experiments, mice were injected with 100 mg/kg salicylate i.p. and were sacrificed 15, 30, 60 or 120 min after injection.

To investigate the effect of methionine, a HOCl scavenger, on the levels of 2,3-DHB and 2,5-DHB, control mice and mice exposed to 5% DSS were given two injections of methionine 30 and 60 min before sacrifice, at 50 or 200 mg/kg i.p., and salicylate was injected 30 min before sacrifice.

To determine if the tissue processing made any contribution to the amount of 2,3-DHB and 2,5-DHB detected, aliquots of homogenate from non-injected mice were spiked with salicylate at 0.25 nmol/mg mucosa (the average amount present in the mucosa 60 min after injection of 100 mg/kg i.p.), re-homogenised, and then processed and analysed as above.

3.2.3.1 HPLC Analyses

HPLC analyses were performed using a Millipore-Waters (MA, USA) HPLC system consisting of 600MS quaternary pump with SILK pulse dampening, 717 refrigerated autosampler, RCM 8 × 10 module with Radial Pak cartridges (10 cm × 8 mm i.d.) and Guard-Pak module. EC detection was used in all assays performed in this thesis, with a dual glassy carbon electrode and Ag/AgCl reference electrode (Bioanalytical Systems Inc., Indiana). Analytical and chromatographic grade solvents were obtained from Millipore-Waters or Ajax. The mobile phases were recirculated in a closed system and sparged continuously with analytical grade helium to exclude oxygen.

For determination of salicylate hydroxylation products, μBondapak C_{18} cartridge plus guard-pak were used with a mobile phase consisting of 30 mM citrate, 30 mM acetate and 40% methanol, pH 3.2, flow rate 1.0 ml/min with SILK pulse dampening on. Fluorescence detection was used to measure salicylate levels, with excitation 300 nm and emission at 415 nm, followed by EC detection of the dihydroxybenzoates, applied potential of +0.7 V, sensitivity 5 namps full scale.

3.2.4 Oxidation of benzoic acids

To determine whether salicylate or its hydroxylation metabolites react with oxidants directly, salicylate, 2,3-DHB and 2,5-DHB (100μM in PBS) were reacted with varying amounts of HOCl (British Drug House (BDH)), H_2O_2 (BDH) and diethylamine NONOate (Cayman Chemical Company) for 30 min, and changes in their fluorescence were measured (Hitachi F-3000 fluorospectrophotometer) at the peak excitation and emission wavelengths for that compound — 2,3-DHB: Ex 310, Em 445; 2,5-DHB: Ex 325, Em 450; salicylate: Ex 300, Em 410. The concentrations of stock solutions of H_2O_2, HOCl and NONOate were checked by UV absorption at 240, 292, and 250 nm respectively.[205–207] NONOate, prepared in sterile PBS pH 8.5, decomposes to release $^{\cdot}NO$ at neutral pH, with a half life of 2.1 min at pH 7.4.[207] Thus 30 min was sufficient for all $^{\cdot}NO$ to be released. The solutions of salicylate and DHBs were also analysed by HPLC for changes in levels of the compounds after oxidant exposure.

3.2.5 Statistical Analysis

Spectrophotometric assays were done in triplicate and all results were standardised to the protein content of the homogenates, using the BioRad micro protein assay. Data are expressed as the mean ± standard deviation and statistical differences were determined using the unpaired Student's *t* test.

3.3 Results

3.3.1 Myeloperoxidase Activity

Details of the observed symptoms and histological appearance of the colon in these mice are described in Chapter 2. Mice exposed to 5% DSS developed diarrhoea and

rectal bleeding, with ulceration and inflammatory infiltrate including neutrophils present in the mucosa. Administration of 3% DSS induced much less severe symptoms, with little rectal bleeding and ulceration. The extent of neutrophil infiltration in these diseases was quantified by determining the mucosal content of MPO. The mean MPO activity in the mucosa increased significantly in both groups of mice exposed to DSS, from 11.3 ± 3.5 μU/g in control mice to 25.2 ± 8.8 μU/g ($P < 0.001$) and 111.9 ± 26.5 μU/g ($P < 0.001$) in mice exposed to 3% DSS for 15 days and 5% DSS for seven days, respectively (Figure 3.2).

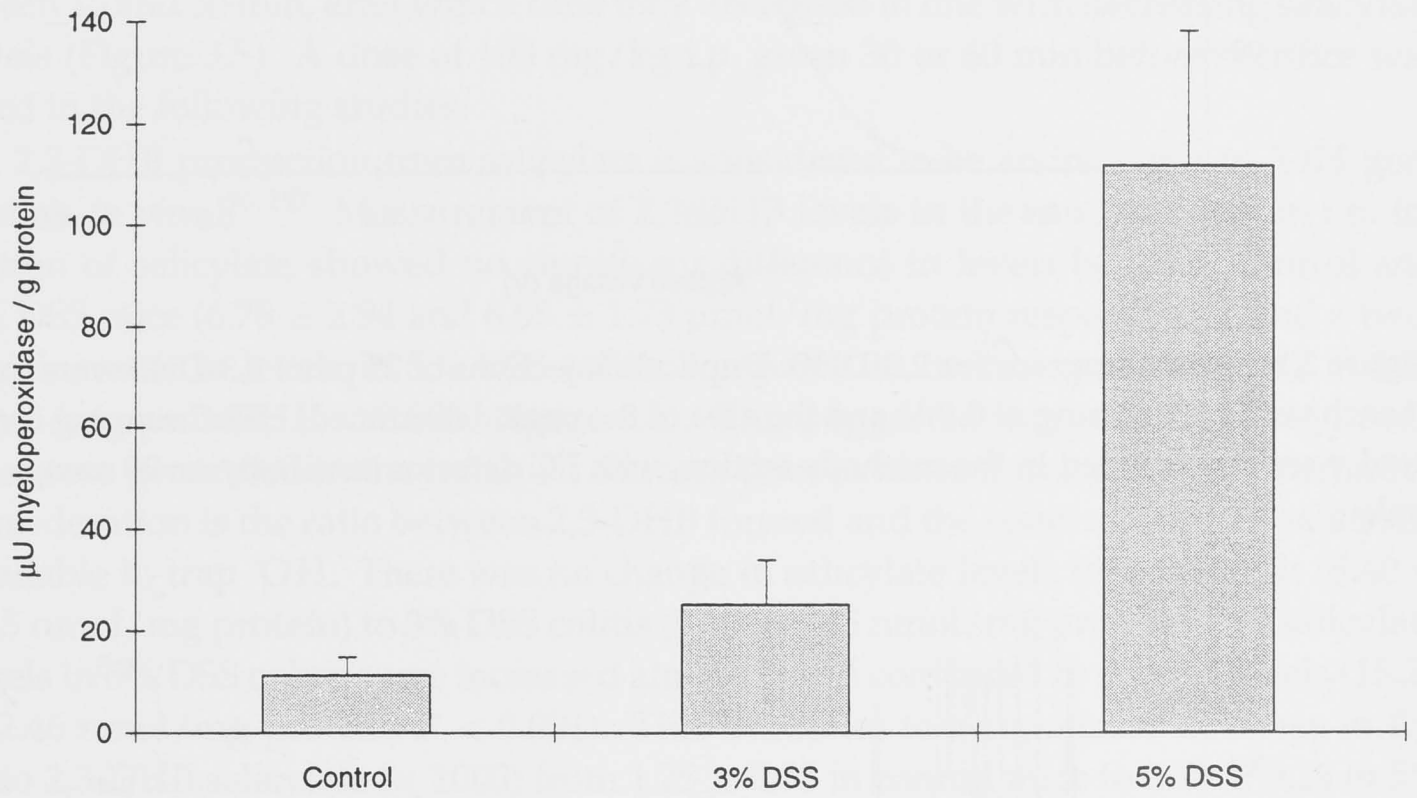


Figure 3.2: Myeloperoxidase activity in the colonic mucosa of control mice (n=24), mice exposed to 3% DSS for 15 days (n=12) or 5% DSS for 7 days (n=10). All groups are significantly different from each other ($P < 0.001$).

3.3.2 Salicylate Hydroxylation

Hydroxylation products in the mucosa from mice injected with salicylate were analysed by HPLC with EC detection. As the amounts of 2,3-DHB to be measured were very small, a voltammogram for 2,3-DHB was constructed to choose the most appropriate voltage which gave maximum response for 2,3-DHB while minimising chromatographic noise (Figure 3.3). A voltage of +0.7 V, which gave 90% of maximum response for 2,3-DHB, was applied for the assays performed in these studies. Using this system, 0.1 pmol 2,3-DHB could be detected readily, and amounts in the range of 0.4–2.0 pmol were measured routinely in samples. Figure 3.4 shows a chromatogram of a solution of standards and a mucosal sample from a mouse injected with salicylate. A recent report cautioned that 2,3-DHB generated during sample collection and processing could make a major contribution to the amount of 2,3-DHB measured

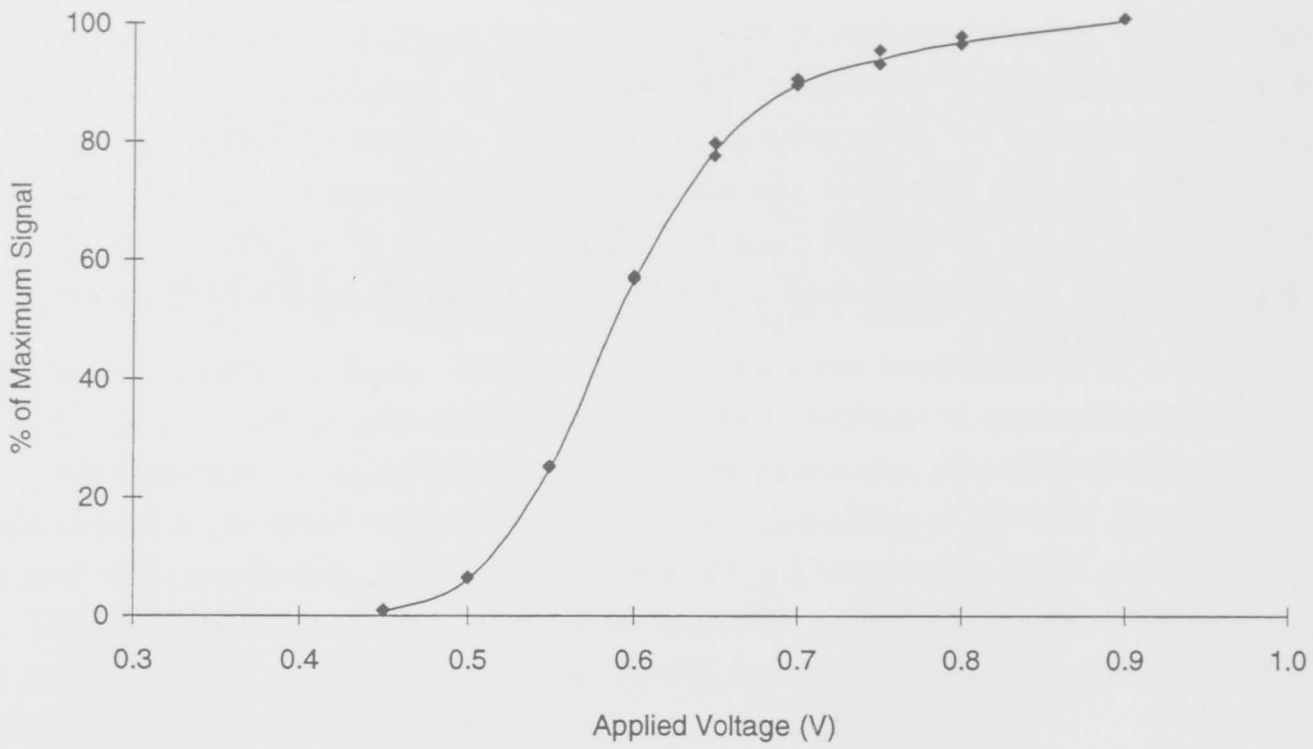


Figure 3.3: Voltammogram for 2,3-DHB. Duplicate injections of 25 pmol 2,3-DHB were made at each voltage, begining at 0.9 V, and the area of the peak determined. HPLC running conditions were as described in the methods section, with EC detector sensitivity on 20 namps full scale.

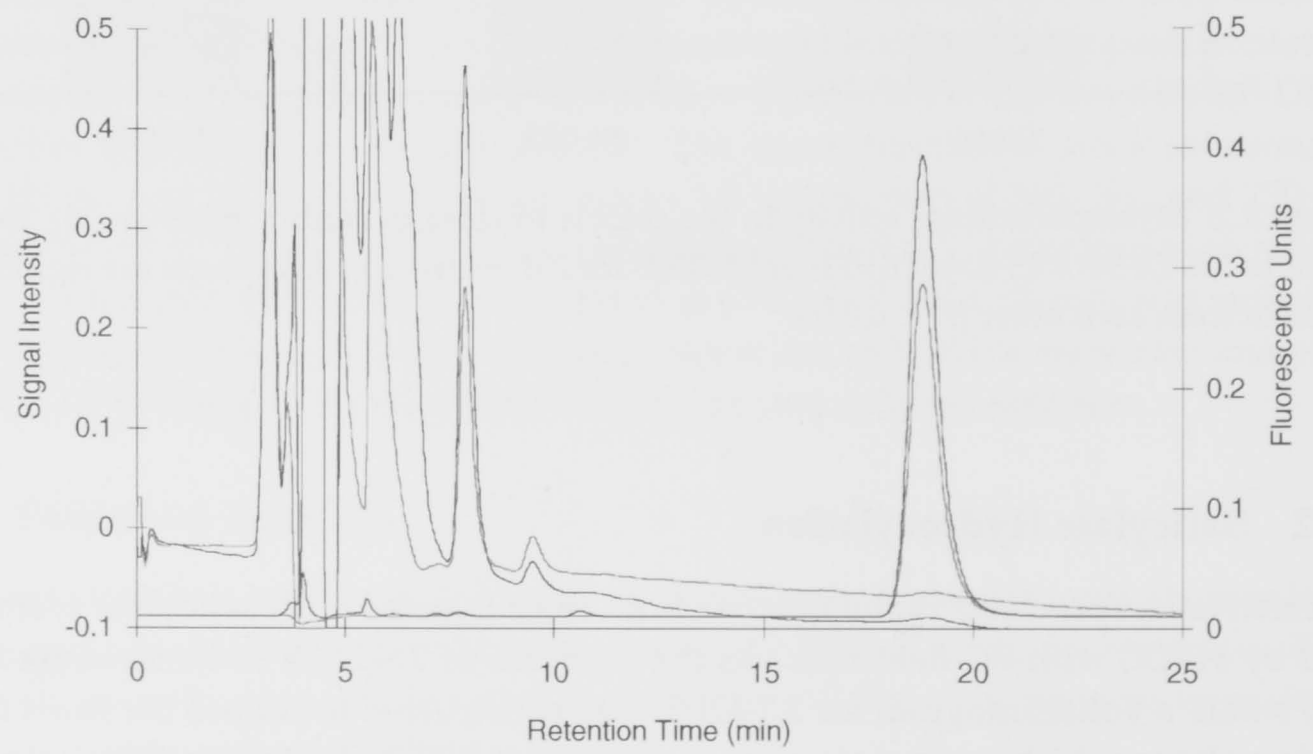


Figure 3.4: Typical chromatograms from analysis of salicylate hydroxylation products. Traces are from EC detector showing 2,5-DHB and 2,3-DHB peaks (retention times of 7.9 and 9.5 min respectively) and from fluorescence detector (FL) showing salicylate peak, (Ex 300, Em 415 nm, retention time 18.8 min) of an injection of a standard solution (EC-green FL-red) and a mouse mucosal sample (EC-black FL-blue). Standard amounts are 1.25 pmol 2,3-DHB, 10 pmol 2,5-DHB and 500 pmol salicylate.

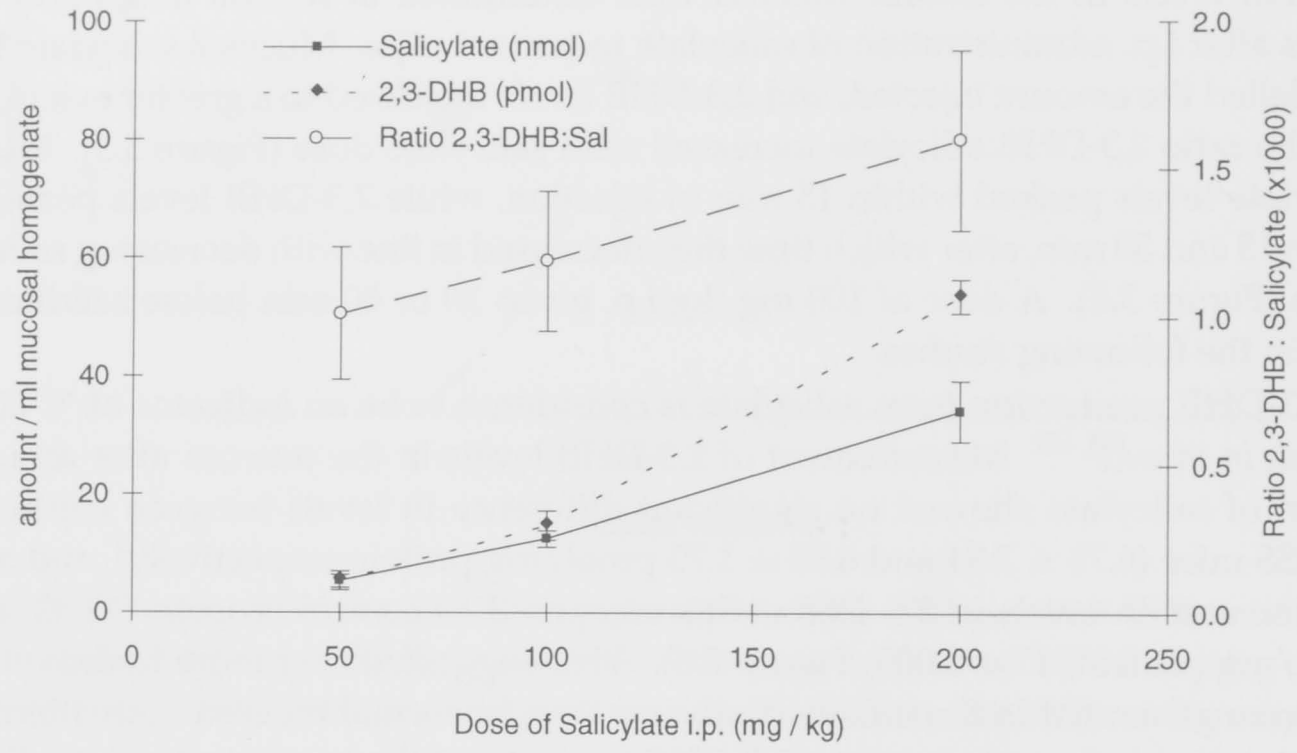
in samples.[208] To address this possibility, mucosa spiked with salicylate *in vitro* was homogenised and analysed for 2,3-DHB. No 2,3- or 2,5-DHB was detected in these samples (data not shown).

To determine an appropriate dose of salicylate and injection time, salicylate and 2,3-DHB levels in the colonic mucosa were determined at several doses and time-points after i.p. administration of salicylate to normal mice. Mucosal salicylate levels parallelled the amount injected, and 2,3-DHB levels increased to a greater extent, such that the ratio 2,3-DHB:salicylate increased with salicylate dose (Figure 3.5). Mucosal salicylate levels peaked within 15 min of injection, while 2,3-DHB levels peaked between 15 and 30 min, after which time they decreased in line with decreasing salicylate levels (Figure 3.5). A dose of 100 mg/kg i.p. given 30 or 60 min before sacrifice was used in the following studies.

2,3-DHB production from salicylate is considered to be an indicator of $^{\cdot}OH$ generation *in vivo*.[195,197] Measurement of 2,3-DHB levels in the mucosa after an i.p. injection of salicylate showed no significant difference in levels between control and 3% DSS mice (6.78 ± 2.94 and 6.55 ± 1.73 pmol/mg protein respectively), and a two-fold increase in levels in 5% DSS colitis compared to control mucosa (12.42 ± 3.65 pmol/mg protein, $P < 0.005$, Figure 3.6). This suggested that more hydroxyl radicals were generated in the inflamed mucosa than in normal mucosa . An alternative consideration is the ratio between 2,3-DHB formed and the concentration of salicylate available to trap $^{\cdot}OH$. There was no change in salicylate levels from controls (5.40 ± 1.45 nmol/mg protein) to 3% DSS colitis (5.50 ± 0.85 nmol/mg protein), but salicylate levels in 5% DSS colitis were increased almost 3-fold compared to control levels (15.24 ± 2.46 nmol/mg protein, $P < 0.001$). This translates to a significant decrease in the ratio 2,3-DHB:salicylate ($\times 1000$) from 1.25 ± 0.33 in control mice to 0.83 ± 0.26 in 5% DSS ($P < 0.005$, Figure 3.7).

A decrease in the ratio 2,3-DHB:salicylate may reflect increased catabolism of the hydroxylated products in the inflamed mucosa. To assess if oxidants may be involved in the catabolism of 2,3-DHB, the effect of oxidant exposure on the fluorescence of 2,3- and 2,5-DHB was investigated *in vitro*. H_2O_2 and $^{\cdot}NO$ had little effect on the fluorescence of 2,3-DHB, with up to a 3-fold excess of oxidant. In contrast, a 3-fold excess of HOCl abolished 93% of the 2,3-DHB fluorescence (Figure 3.8). The 2,5-DHB isomer was more sensitive to oxidation, with a 1.5-fold excess of HOCl being sufficient to abolish 96% of fluorescence and a 3-fold excess of $^{\cdot}NO$ causing a 8% decrease (Figure 3.8). HPLC analysis confirmed these results, with the decreased fluorescence corresponding to decreased EC peak areas. No new peaks were detected in these samples either electrochemically or by fluorescence (data not shown).

To investigate the possibility of HOCl oxidation of 2,3-DHB *in vivo*, mice exposed to 5% DSS were injected with the HOCl scavenger methionine prior to, and coincident with, salicylate. Methionine at 50 mg/kg i.p. had no effect on 2,3-DHB levels or on the ratio of 2,3-DHB:salicylate in the colonic mucosa in control or DSS mice, while at 200 mg/kg, methionine resulted in a significant decrease in both these measurements (Figure 3.9). While 2,5-DHB levels were more than 15-fold 2,3-DHB levels (137 ± 49 pmol/mg protein compared to 8.5 ± 3.4 pmol/mg), similar changes were



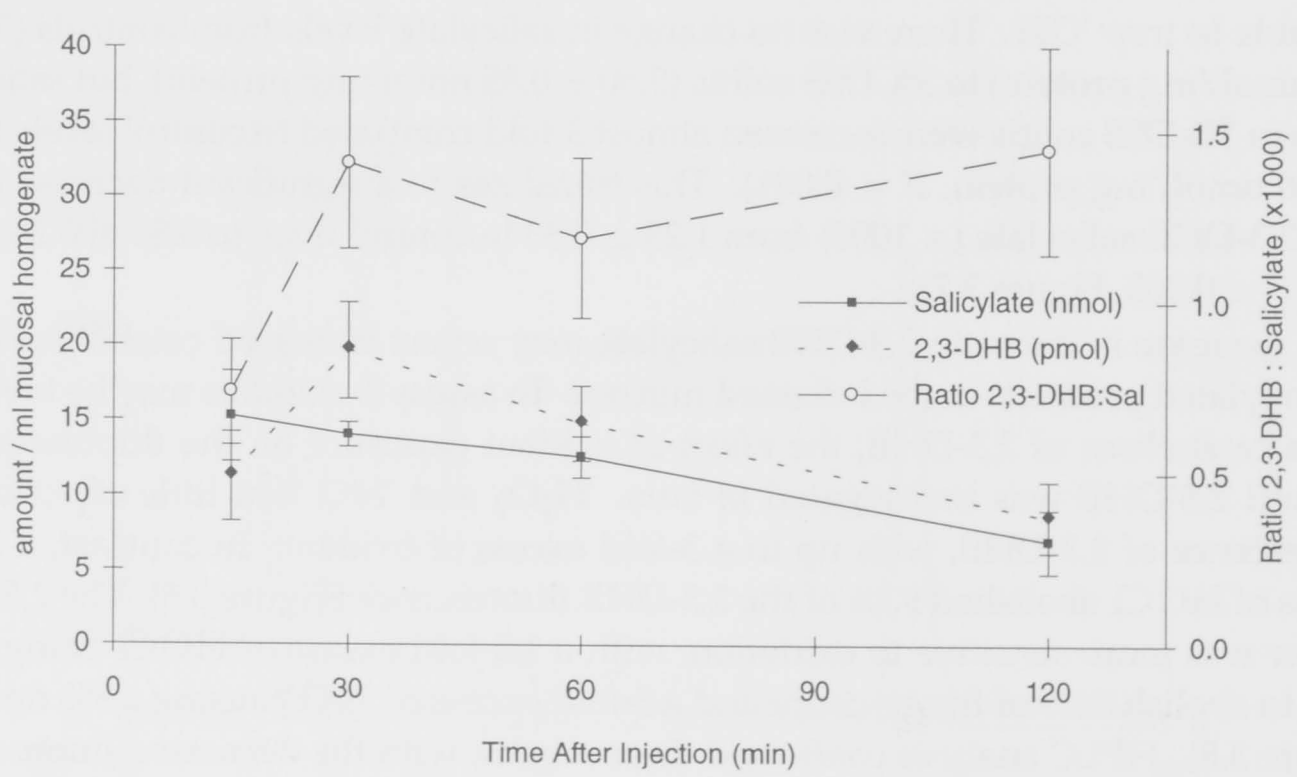


Figure 3.5: 2,3-DHB and salicylate levels in the mucosa of healthy mice. Levels 60 min after i.p. injection of various doses is shown (top) and the time course after i.p. injection of 100 mg/kg (bottom). $3 \leq n \leq 5$ mice for each point.

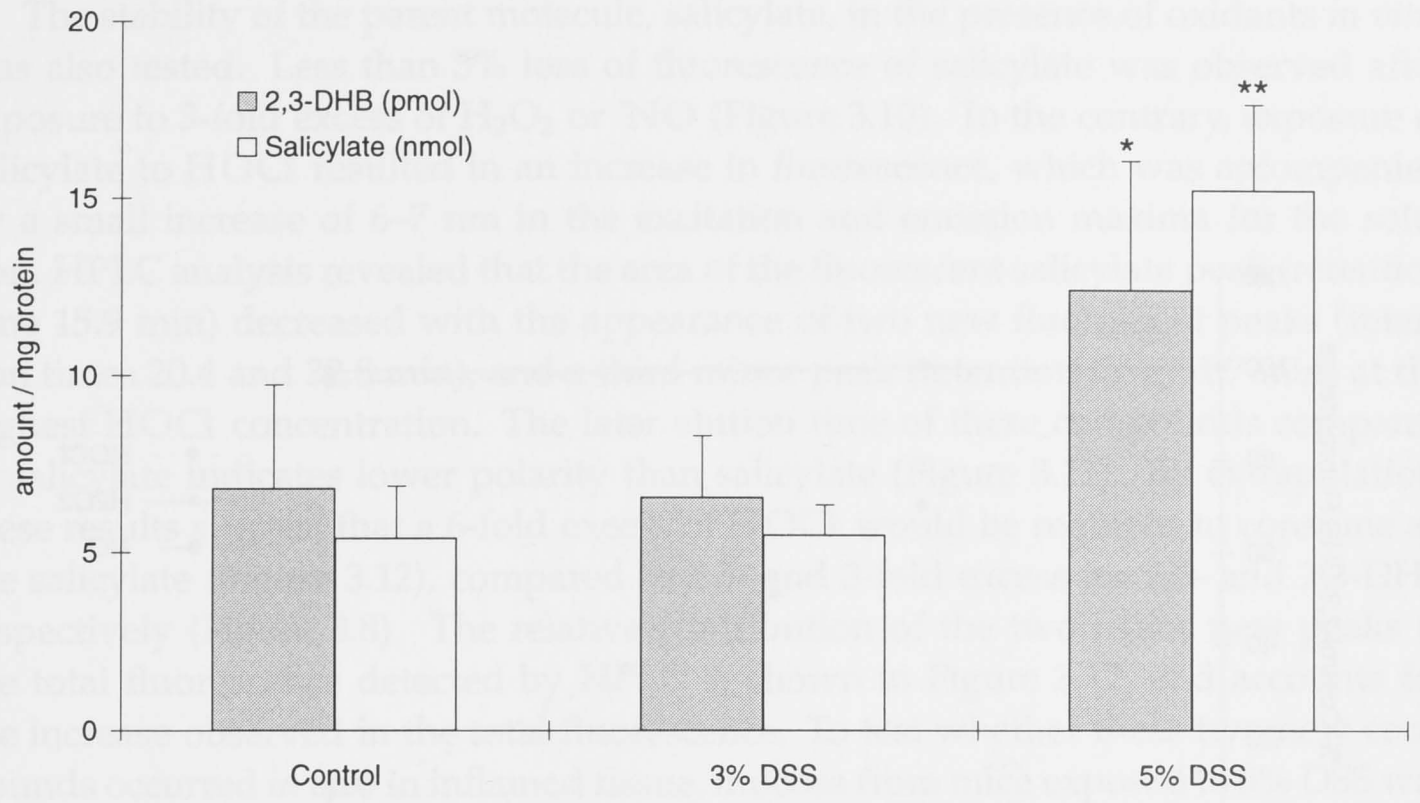


Figure 3.6: Mucosal levels of 2,3-DHB and salicylate in the colonic mucosa of control mice (n=15) and mice treated with 3% DSS for 15 days (n=6) or 5% DSS for 7 days (n=8). *$P < 0.005$ and **$P < 0.001$ compared to both control and 3% DSS levels of the same compound.

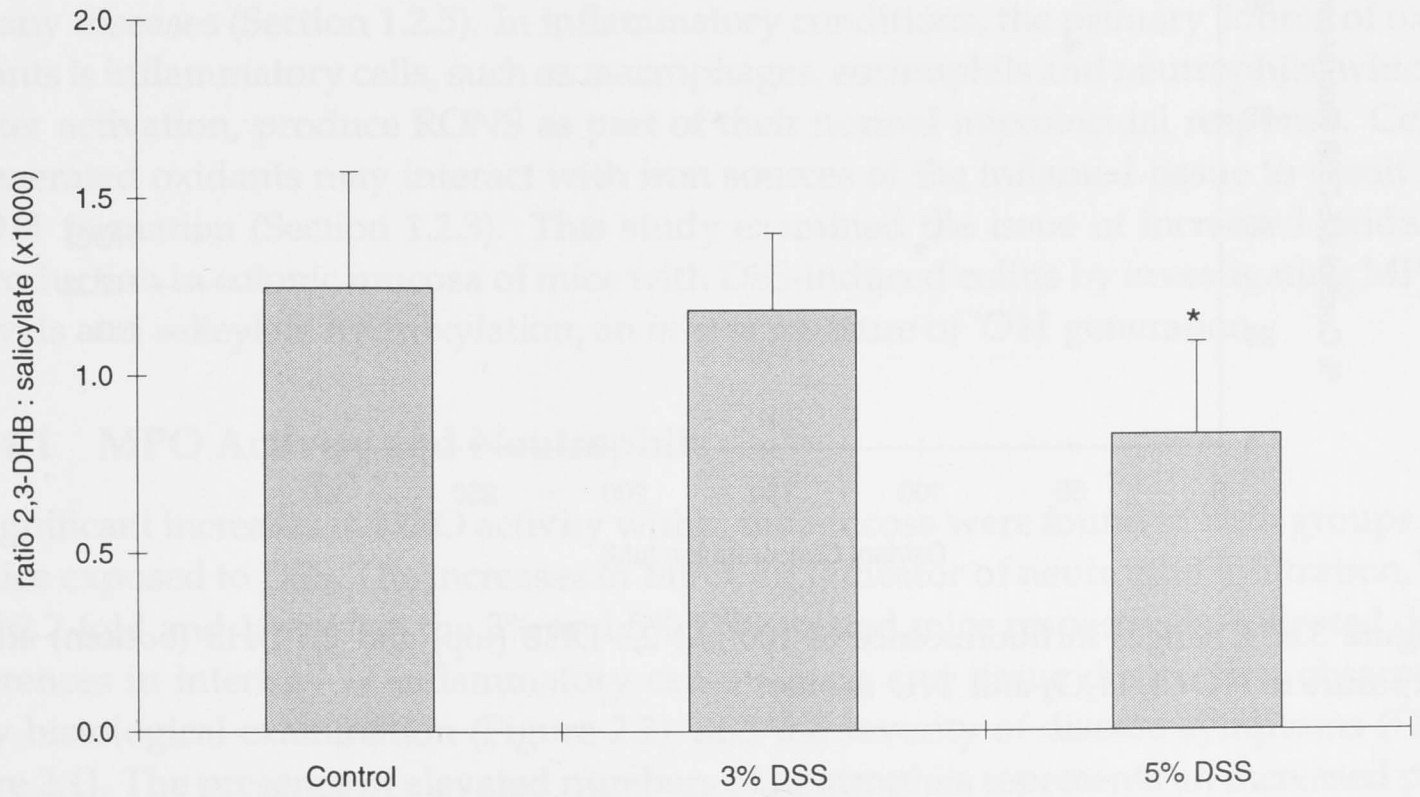
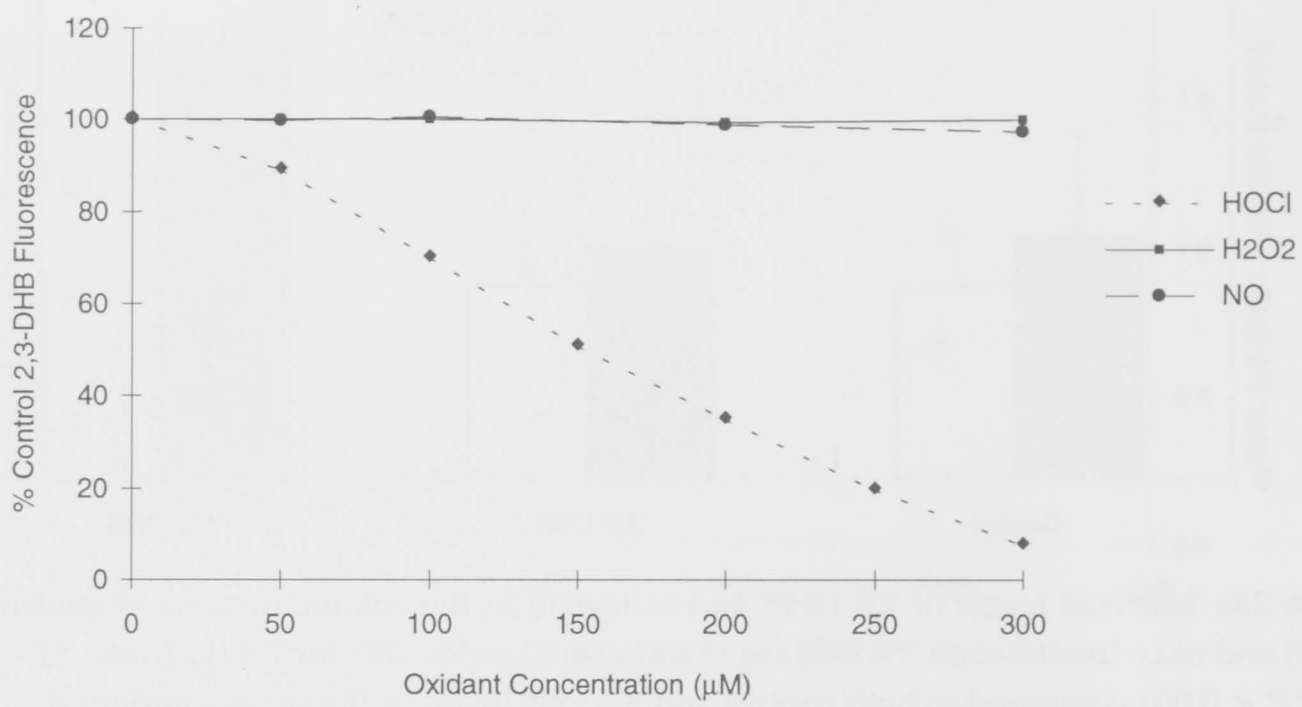


Figure 3.7: Ratio of 2,3-DHB:salicylate in the colonic mucosa of control mice (n=15) and mice treated with 3% DSS for 15 days (n=6) or 5% DSS for 7 days (n=8). *$P < 0.005$ and $P < 0.05$ compared to control and 3% DSS respectively.



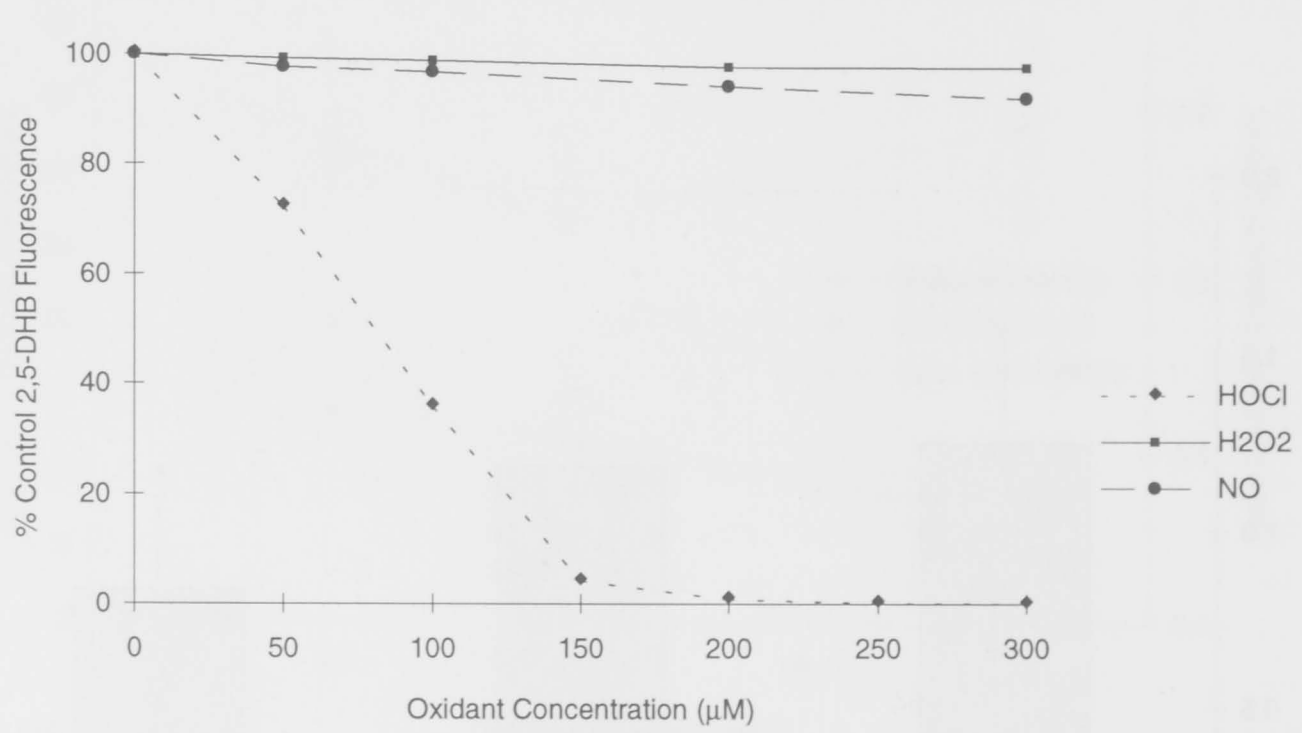


Figure 3.8: Changes in fluorescence of 100 μM 2,3-DHB (top) and 2,5-DHB (bottom) after exposure to HOCl, H_2O_2 and $^{\bullet}NO$ *in vitro*.

observed in the ratio of 2,5-DHB:salicylate in these mice (Figure 3.9).

The stability of the parent molecule, salicylate, in the presence of oxidants *in vitro* was also tested. Less than 5% loss of fluorescence of salicylate was observed after exposure to 3-fold excess of H_2O_2 or $^{\bullet}NO$ (Figure 3.10). To the contrary, exposure of salicylate to HOCl resulted in an increase in fluorescence, which was accompanied by a small increase of 6–7 nm in the excitation and emission maxima for the solution. HPLC analysis revealed that the area of the fluorescent salicylate peak (retention time 15.9 min) decreased with the appearance of two new fluorescent peaks (retention times 20.4 and 32.8 min), and a third minor peak (retention time 51.7 min) at the highest HOCl concentration. The later elution time of these compounds compared to salicylate indicates lower polarity than salicylate (Figure 3.11). By extrapolation, these results suggest that a 6-fold excess of HOCl would be required to consume all the salicylate (Figure 3.12), compared to 1.5- and 3-fold excess for 2,5- and 2,3-DHB respectively (Figure 3.8). The relative contribution of the two major new peaks to the total fluorescence detected by HPLC is shown in Figure 3.12, and accounts for the increase observed in the total fluorescence. To test whether these two new compounds occurred *in vivo* in inflamed tissue, mucosa from mice exposed to 5% DSS was re-analysed with maximum fluorescence sensitivity. The peaks were not detected in these samples when the fluorescence detector was operated at maximum gain.

3.4 Discussion

Increased production of oxidants is believed to be involved in the tissue damage of many diseases (Section 1.2.5). In inflammatory conditions, the primary source of oxidants is inflammatory cells, such as macrophages, eosinophils and neutrophils, which, after activation, produce RONS as part of their normal microbicidal response. Cell-generated oxidants may interact with iron sources of the inflamed tissue to result in $^{\bullet}OH$ formation (Section 1.2.3). This study examined the issue of increased oxidant production in colonic mucosa of mice with DSS-induced colitis by investigating MPO levels and salicylate hydroxylation, an *in vivo* measure of $^{\bullet}OH$ generation.

3.4.1 MPO Activity and Neutrophils

Significant increases in MPO activity within the mucosa were found in both groups of mice exposed to DSS. The increases in MPO, an indicator of neutrophil infiltration,[191] of 2.2-fold and 10-fold in the 3% and 5% DSS-treated mice respectively, reflected differences in intensity of inflammatory cell infiltrate and tissue destruction observed by histological examination (Figure 2.2), and the severity of disease symptoms (Figure 2.1). The presence of elevated numbers of neutrophils represents an increased potential for oxidant production, not only of HOCl generated by MPO, but also H_2O_2, $^{\bullet}NO$, and $ONOO^-$, which neutrophils are capable of producing (Section 1.2.3).

Neutrophils have been shown to be central to the development of severe colitis in this model by the exposure of neutrophil-depleted mice[179] and rats[209] to DSS. Depletion of neutrophils, by treatment with anti-neutrophil antibody, dramatically re-

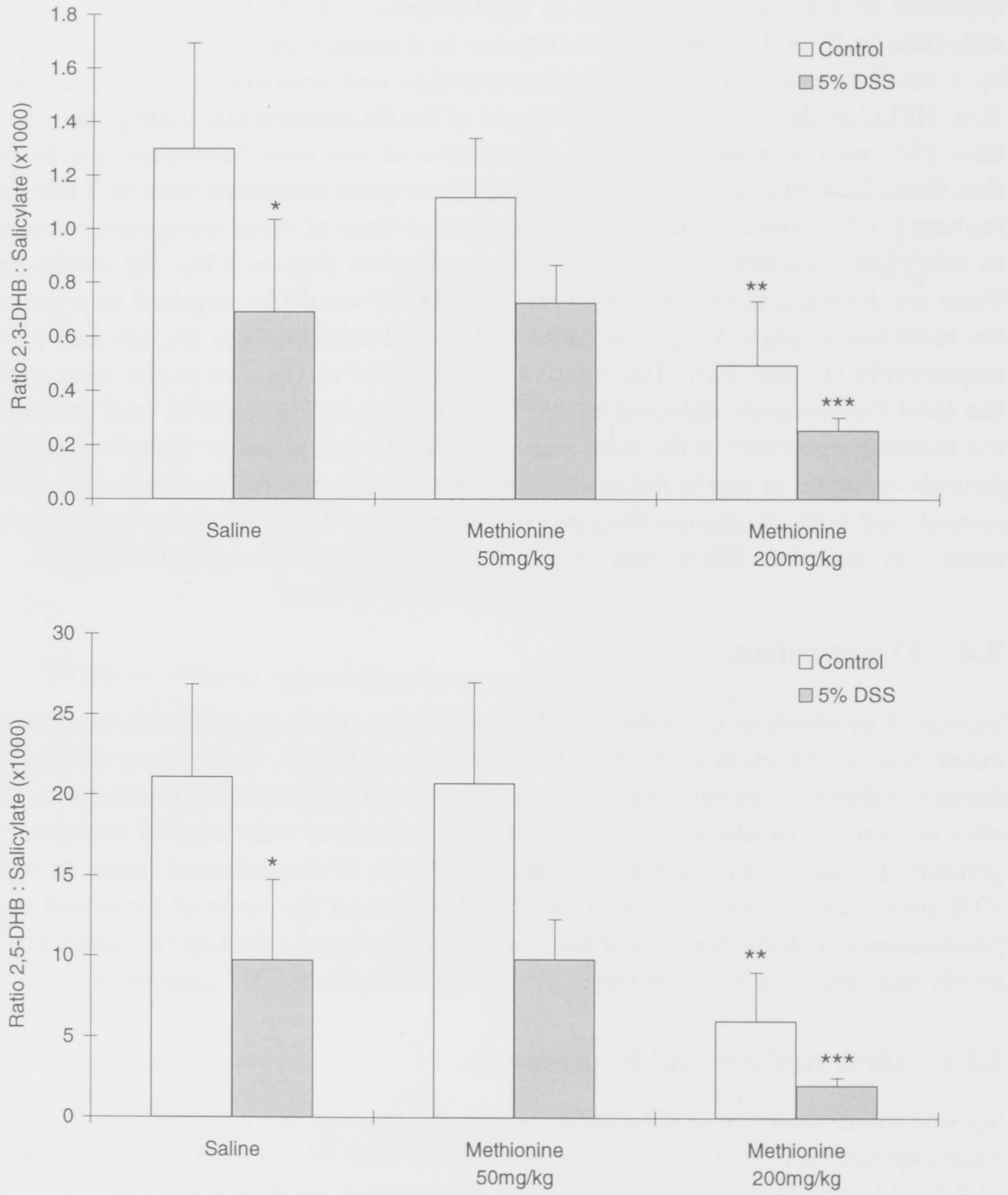


Figure 3.9: Effect of methionine on mucosal ratios of 2,3-DHB:salicylate (top) and 2,5-DHB:salicylate (bottom) in colitis induced with 5% DSS. * $P < 0.05$ and ** $P < 0.01$ compared to saline-injected control group. *** $P < 0.05$ compared to saline-injected DSS group. n=5 each group.

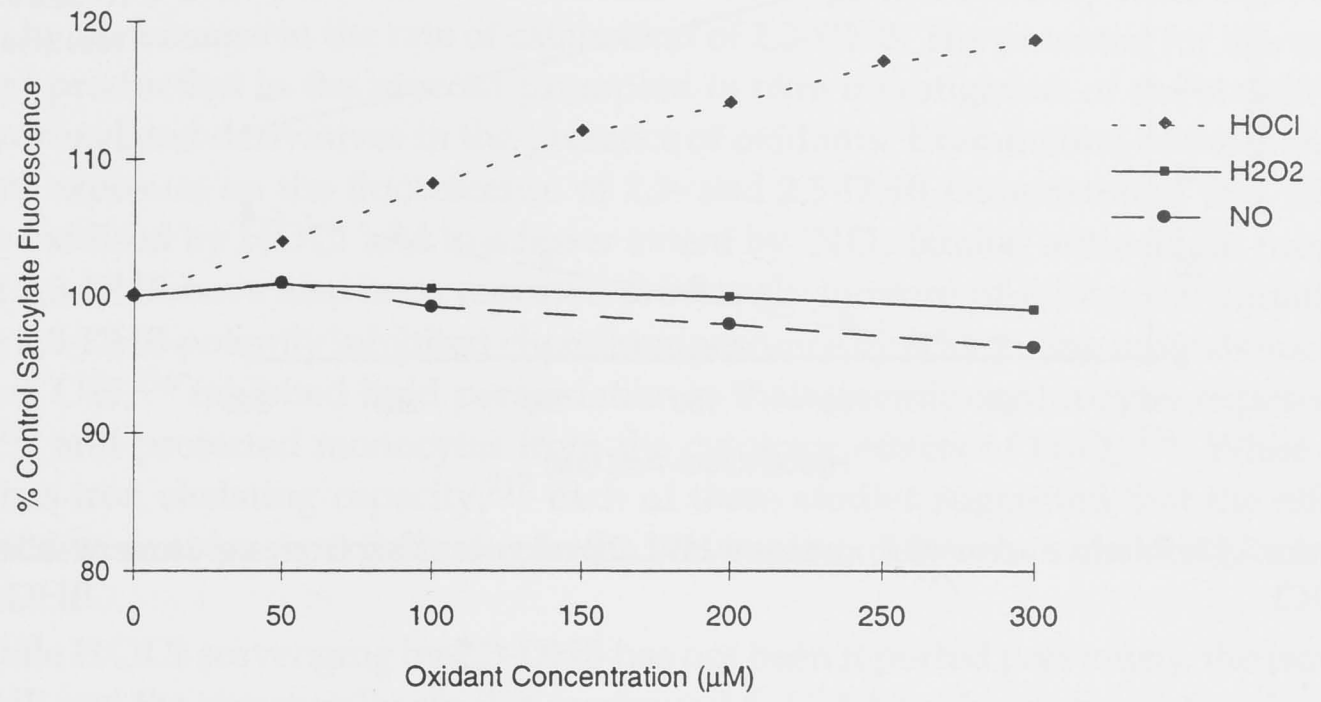


Figure 3.10: Fluorescence of salicylate (100 μM) after exposure to HOCl, H_2O_2 and $^{\bullet}NO$ *in vitro*.

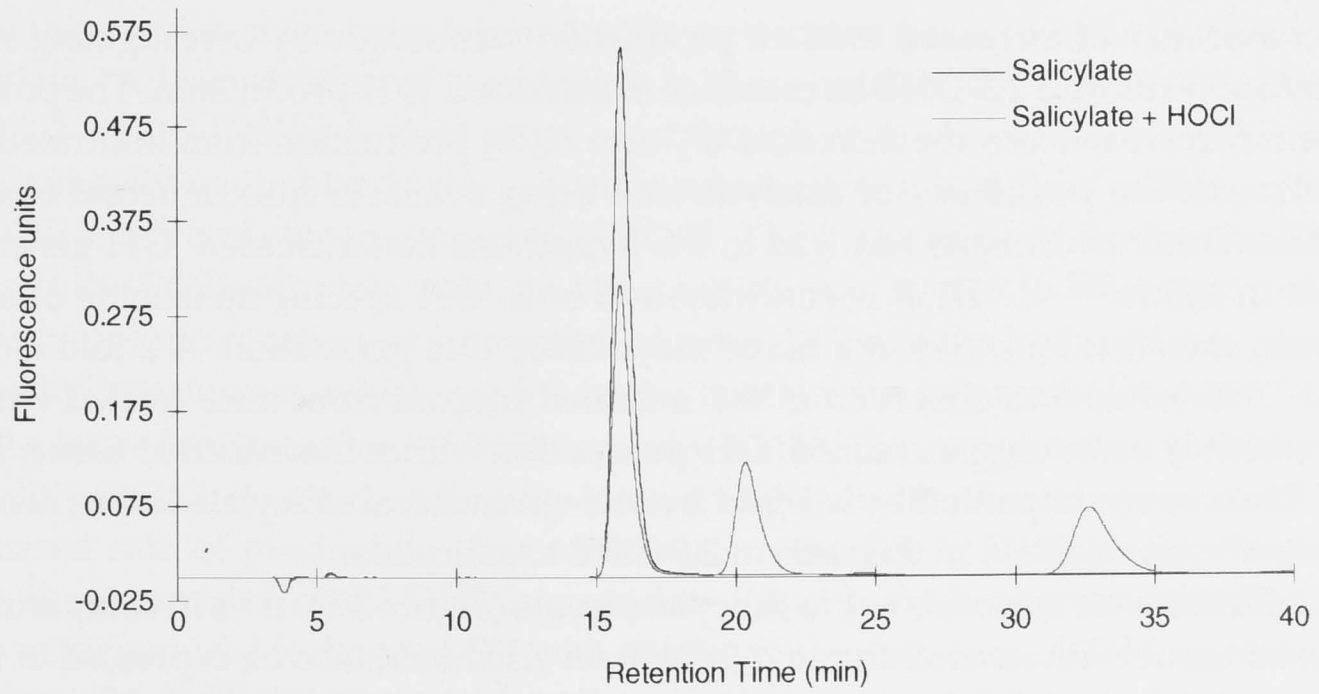


Figure 3.11: Chromatogram of salicylate (retention time 15.9 min), 100μM, before (black) and after (red) exposure to 250μM HOCl. Retention times of the new peaks are 20.4 and 32.8 min.

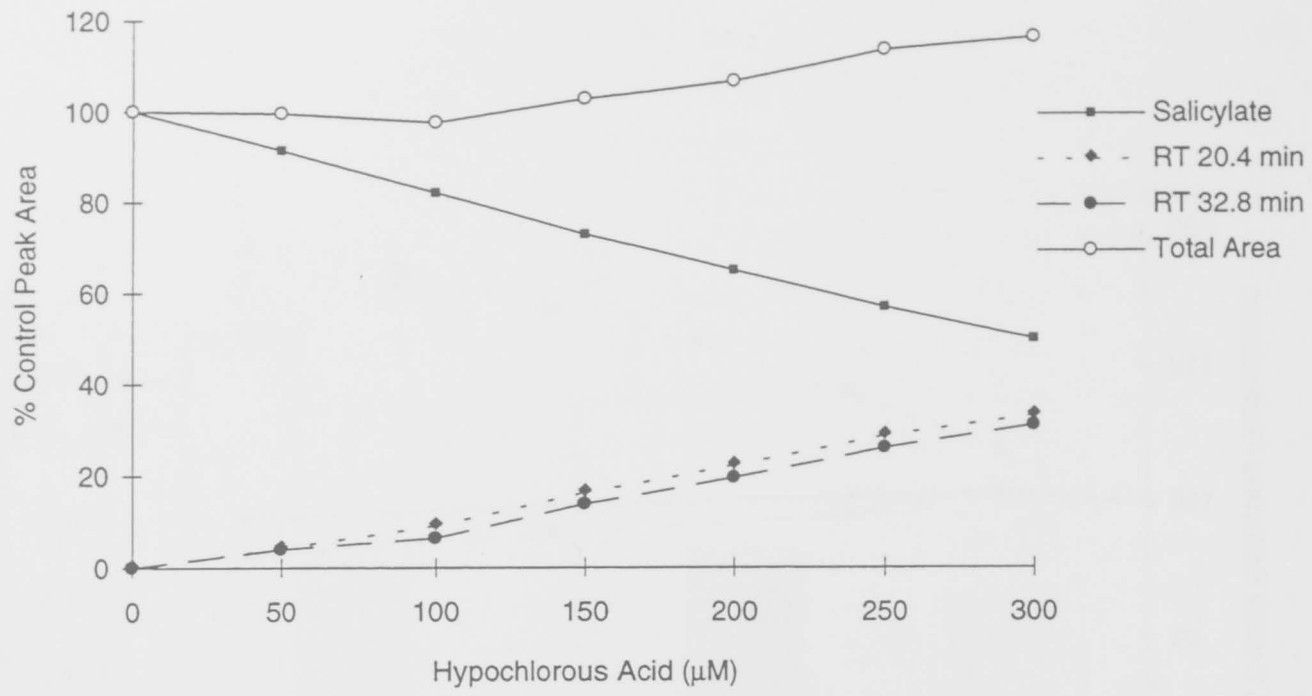


Figure 3.12: Relative areas of fluorescent HPLC peaks resulting from exposure of salicylate to HOCl.

duced the occurrence of rectal bleeding in mice, and attenuated mucosal permeability changes and epithelial cell denudation in rats. These results demonstrate that the presence of neutrophils is important in the pathogenesis of acute DSS-induced colitis.

3.4.2 Salicylate Hydroxylation

Direct evidence of increased oxidant production was sought by investigating salicylate hydroxylation to 2,3-DHB as evidence of increased $^{\cdot}OH$ production. The potential in the inflamed mucosa for increased $O_2^{\cdot-}$ and H_2O_2 production from infiltrated neutrophils and the possibility of catalytic iron being available from mucosal bleeding and from luminal contents has lead to the hypothesis that increased $^{\cdot}OH$ generation occurs in colitis.[137] 2,3-DHB is considered to be a $^{\cdot}OH$ specific metabolite of salicylate, and therefore its levels in a tissue may reflect $^{\cdot}OH$ generation. A 2-fold increase in 2,3-DHB levels was observed in the inflamed mucosa from mice treated with 5% DSS, possibly indicating increased $^{\cdot}OH$ production within the inflamed tissue. However, this was accompanied by a 3-fold increase in mucosal salicylate levels, resulting in a significant decrease in the ratio of 2,3-DHB to salicylate.

2,3-DHB levels were related to salicylate levels (Figure 3.5), thus in comparing the diseased tissue with normal tissue, 2,3-DHB levels should also be expressed in terms of the amount of salicylate present. The use of the ratio DHB:salicylate has been discussed by other investigators where changes in blood-brain barrier integrity or acute inflammation has affected salicylate levels in the brain or lung respectively.[203,210,211] Increased mucosal vascular permeability has been reported in several animal models of intestinal inflammation[170,212–214] and is a likely explanation for the elevated mucosal salicylate levels in DSS-induced colitis. However, if $^{\cdot}OH$ generation was

unchanged or increased, this should have been accompanied by similarly increased 2,3-DHB levels.

The observation that the ratio 2,3-DHB:salicylate decreased in the mucosa of DSS-induced colitis could be the result of decreased production or increased catabolism of the hydroxylated products. An increase in $^{\bullet}OH$ generation may have been concealed by an increase in the rate of catabolism of 2,3-DHB. The potential for increased oxidant production in the mucosa prompted *in vitro* investigation of the stability of the hydroxylated derivatives in the presence of oxidants. Examination of the effect of oxidant exposure on the fluorescence of 2,3- and 2,5-DHB demonstrated that DHBs can be oxidised by HOCl and to a lesser extent by $^{\bullet}NO$. Similar antioxidant properties of 2,3-DHB have also been reported previously in more physiological situations where 2,3-DHB potently inhibited chemiluminescence by scavenging radicals such as $O_2^{\bullet-}$ and $^{\bullet}OH$,[116] inhibited lipid peroxidation in thalassaemic erythrocytes exposed to H_2O_2[215] and protected monocytes from the cytotoxic effects of H_2O_2.[216] While 2,3-DHB has iron chelating capacity,[217] each of these studies suggested that the effects observed were at least partially due to the scavenging, rather than chelating, activity of 2,3-DHB.

While HOCl scavenging by 2,3-DHB has not been reported previously, the isomer 2,5-DHB and the structurally similar compound 5-ASA have been shown to scavenge HOCl.[162,198] Furthermore, the hydroxylation of salicylate to 2,5-DHB by stimulated neutrophils was found to be enhanced by the HOCl scavenger methionine[198] and glutathione[218] indicating that the 2,5-DHB measured was the net accumulation, not the total, 2,5-DHB produced.[198] The situation *in vivo* in an acute neutrophilic inflammation such as DSS-induced colitis may be similar, and thus administration of a HOCl scavenger may enhance 2,3- and 2,5-DHB levels measured in the mucosa. To investigate this *in vivo*, mice were injected with methionine prior to and coincident with salicylate. Administration of methionine in these animals did not increase the mucosal 2,3- or 2,5-DHB:salicylate ratio, even though at the lower dose, methionine is approximately equimolar to the salicylate administered, and thus well in excess of 2,3-DHB levels and able to compete for HOCl. The higher dose of methionine further reduced the DHB:salicylate ratio, an effect which may be due to scavenging of $^{\bullet}OH$ at this high concentration, or promotion of methylation of the hydroxylated products by methyltransferases, which use *S*-adenosylmethionine as a methyl donor.

The lack of enhancement of DHB levels by methionine suggests that in DSS-induced colitis HOCl is not contributing to an increased rate of catabolism of DHB. A decreased rate of production may contribute to the fall in DHB levels. In normal mice, the generation of 2,3-DHB is considered due to the background level of $^{\bullet}OH$ from mitochondrial leakage of $O_2^{\bullet-}$ with subsequent generation of $^{\bullet}OH$ by metal ions (Section 1.2.1.1).[193] Studies performed in Chapter 4 suggest the possibility of mitochondrial dysfunction in the mucosa of DSS-exposed mice, due to severe depletion of ubiquinol-9, an element of the electron transport chain (Section 1.2.2), which may alter the level of background $^{\bullet}OH$ generation, thus contributing to decreased 2,3-DHB production. The decrease in 2,5-DHB:salicylate ratio may be contributed to by decreased systemic cytochrome P-450 activity, which is diminished in adjuvant arthritis[219] and

other models of inflammation and infection.[220–222]

Thus, the study of salicylate hydroxylation has not provided evidence to support a role for ·OH in the tissue injury of DSS-induced colitis. While this method has been informative in many situations, including *in vitro* studies on ·OH generation[223,224] and ischaemic-reperfusion studies on isolated organs,[199,200,225] there are few attempts[203] to apply salicylate hydroxylation to *in vivo* situations of inflammation. Decreased production of 2,3-DHB has not been previously reported in salicylate hydroxylation studies, and it is possible that changes in other metabolic pathways relevant to DHB levels are altered in this disease, thus masking any changes that may be due to alterations in the ·OH flux. Methodology may be improved by avoiding systemic administration of salicylate, as there may be a significant systemic contribution to the amount of DHB measured in the tissue of interest.[210] Local administration, for instance luminally in an occluded segment of colon in the anaesthetised animal, may reduce the problems of the differential distribution and changes in hepatic metabolism. Several investigators have used ·OH scavengers to prevent 2,3-DHB formation, increasing the specificity of changes observed.[202,226] These approaches may improve results obtained with this method, especially in disease states which alter many aspects of the physiology and biochemistry of an animal.

Considerable evidence supports a possible role for ·OH in the pathogenesis of colitis. In animals, the iron chelator, desferrioxamine, and the ·OH scavenger, dimethylsulfoxide, significantly attenuated fMLP-induced mucosal damage,[170] although these agents did not decrease the severity of inflammation in acetic acid-induced colitis.[168] The ability of 5-ASA to scavenge ·OH[116,164] and, in humans, the finding of a metabolite of 5-ASA in the faecal material of IBD patients that is also formed by irradiation of 5-ASA *in vitro* suggests that ·OH are generated in the inflamed colon and that scavenging of RONS by 5-ASA may contribute to its efficacy.[150] Should iron availability be involved in ·OH-mediated injury, there would be potential for modulating iron levels within the colon or faecal stream of patients to reduce ·OH generation. Further investigation into the possible involvement of iron- and ·OH-mediated tissue injury in colitis is required to fully explore this possibility.

3.4.3 Reaction of Salicylate and HOCl

Salicylate was shown to react with HOCl, resulting in the production of two major and one minor fluorescent products of lower polarity than salicylate (Figure 3.11). The formation of such products was also observed by Davis *et al.* using UV absorption for detection, rather than fluorescence.[227] While a detailed examination of these compounds was outside the scope of this study, we speculate that these compounds may be dimers, or larger polymers, of salicylate formed *via* the salicyl radical, with isomerization, diradical reaction and enolization, as has been described for the formation of dimers of the phenolic amino acid tyrosine.[228] *

Dityrosine formation is an indicator of protein oxidation[228] and has been shown to occur in fibronectin[229] and low density lipoprotein after exposure to HOCl.[230] This suggests that HOCl may be capable of initiating the dimerization of salicylate and

*The unknown fluorescent compounds could also be chlorinated derivatives of salicylate, although this would result in products of greater polarity which would be expected to elute before salicylate.

the presence of salicylate dimers *in vivo* may indicate increased oxidant production. However, using the salicylate hydroxylation tissue preparation and HPLC procedure, these products were not detected in the mucosa of mice with DSS-induced colitis. Similarly, Davis *et al.* did not report finding these metabolites after exposure of salicylate to stimulated neutrophils.[227] However, neither extraction nor detection methods were optimised for these compounds, and the UV and fluorescence maxima may be quite different from those of salicylate. While they will not be major products from salicylate *in vivo*, if the reaction rate of salicylate with HOCl resembles that of the tyrosine-dityrosine reaction, then they may similarly be a useful indicator of oxidant production *in vivo*.

Examination of HOCl involvement in the inflammation of IBD by the investigtion of novel metabolites of the scavenger 5-ASA has been attempted previously. 5-ASA inhibits the luminol-dependent chemiluminescence of stimulated neutrophils by scavenging of oxidants including HOCl.[162] The reaction of 5-ASA with HOCl was found to result in a non-fluorescent product, which was later indentified as 5-nitroso-salicylate.[231] Attempts were then made to detect this compound in the faecal material of IBD patients, however, the abundance of other fluorescent compounds in faecal material, and the low levels of this metabolite, made HPLC attempts futile. Measurement of this metabolite with antibodies may have been possible, however attempts to raise an antibody to this compound were also unsucessful (M. B. Hallett, personal communication).

3.4.4 Conclusion

The mucosal MPO activity in mice exposed to DSS is significantly higher than in normal mice, indicating the possible role of neutrophils and their oxidants in the pathogenesis of this disease. Salicylate hydroxylation studies revealed a decrease in the ratio DHB:salicylate, possibly indicating greater catabolism or decreased production of 2,3-DHB. Thus it was not clear whether the amount of $^{\cdot}OH$ generated had decreased, or if other metabolic changes were obscuring the measurement of $^{\cdot}OH$ by this method. While HOCl could oxidise DHB *in vitro*, HOCl did not appear to be involved in catabolism of DHB *in vivo* in DSS-induced colitis.

Chapter 4

Mucosal Antioxidant and Thiol Status

4.1 Introduction

Antioxidants are the first line of defence against excess oxidant production. They include molecules such as ascorbate, urate, ubiquinol and tocopherol, which operate in the different chemical phases of the cellular material to scavenge radicals and oxidants and prevent more serious damage (Section 1.2.2). By definition, antioxidants react preferentially with oxidants, preserving other surrounding molecules from damaging oxidation, thus the depletion of antioxidants would be one of the first events in oxidative injury. In IBD, studies in our laboratory have shown that ascorbate levels are significantly decreased,[141] and that there is depletion of other antioxidant defences, including urate, glutathione and ubiquinol-10, in the inflamed mucosa of IBD patients.[142,143]

Such a compromise of the defences would leave the tissue vulnerable to further oxidant stress. Thiols are very susceptible to oxidation and thus may also be a sensitive marker of oxidative injury. Many proteins contain thiols which are important for enzymic activity and structure, and thus may be inactivated by oxidation.[102,232,233] GAPDH is one such protein[234,235] which has been proven to be particularly sensitive to oxidation.[103,236–238] Reduced protein thiols react irreversibly with [^{14}C]-iodoacetamide ([^{14}C]-IAM) and subsequently can be identified by gel electrophoresis and autoradiography, while oxidised protein thiols remain unlabelled. Previous work in our laboratory has shown oxidation of GAPDH thiols and loss of GAPDH activity in the colonic epithelial cells from inflamed, but not non-inflamed, mucosa of IBD patients, providing direct evidence of oxidative tissue injury in IBD.[149]

In this study, changes in the antioxidant status in the colonic mucosa of mice with DSS-induced colitis were characterised, and one potential target for oxidant injury was examined. The capacity of the mucosa to scavenge free radicals was assessed, followed by analysis of the major components of the lipophilic and aqueous antioxidant defences. Further, the total reduced thiol content of the mucosa was determined, giving an overall indication the thiol status, and the GAPDH activity of the mucosa and the [^{14}C]-IAM binding of mucosal proteins were examined as indicators of protein thiol status.

4.2 Methods

4.2.1 Dextran Sulfate-Induced Colitis

Colitis was induced in 5–6 week old male CBA/H mice by administering distilled water supplemented with 5% DSS *ad libitum* for 8 days (Section 2.2.1). Mice were observed daily for fluid intake and for the major symptoms of diarrhoea and rectal bleeding, and disease course details can be seen in Appendix A. Colonic mucosa was collected as described previously (Section 2.2.2), and analysed as soon as possible for the antioxidant indicators below. MPO activity of the mucosa was also determined according to the method in Section 3.2.2.

4.2.2 Total Scavenging Capacity

Total aqueous peroxyl radical scavenging capacity was assessed by inhibition of 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH)-induced loss of phycoerythrin fluorescence according to Glazer[239] with modifications, as previously reported.[142] This method measures the capacity of the sample to inhibit oxidative damage to phycoerythrin by peroxyl radicals generated by the thermal decomposition of AAPH (Polysciences Inc.), thereby causing a lag phase in the loss of endogenous phycoerythrin fluorescence. The reaction mixture contained 20 nM phycoerythrin, 10 mM AAPH in chelex-100 treated PBS, and 15,000×g supernatant of homogenates. Baseline phycoerythrin fluorescence in the presence of sample was monitored for 3–5 min before the reaction was initiated by the addition of AAPH. The fluorescence of phycoerythrin was monitored continuously for 15–30 min. Measurements were carried out in an Hitachi F-3000 fluorospectrophotometer, maintained at 37°C and fitted with an electronic cuvette stirrer, with excitation and emission wavelengths of 540 nm and 575 nm respectively. Total scavenging capacity was defined as the lag phase in minutes from the addition of AAPH to the commencement of fluorescence decay.

4.2.3 Analyses of Antioxidant Content

Analyses of the major lipophilic and aqueous antioxidants were performed by HPLC with EC detection using the system described in Section 3.2.3.1. HPLC standards for uric acid and ubiquinol-10 were obtained from Sigma, and α- and γ-tocopherol, ubiquinol-9 and ascorbate were obtained from Kodak (New York), Fluka (Switzerland), and Aldrich (Wisconsin) respectively.

4.2.3.1 Lipophilic Antioxidants

Simultaneous determination of reduced ubiquinols and tocopherols was performed based on the methods of Lang *et al.*[240] and Matsura *et al.*,[241] as previously reported.[142] Butylated hydroxytoluene was added to homogenate aliquots before storage to prevent autoxidation. Aliquots were diluted to 1 ml with cold HBSS and tocopherols and ubiquinols extracted with a mixture of sample:ethanol:methanol:*n*-hexane of 1:1:1:2

(v/v). The *n*-hexane layer was dried under nitrogen gas, the residue redissolved in 100 μL ethanol:methanol (1:1) and 60 μL injected. HPLC was performed with a Nova-Pak C_{18}, 4 μm cartridge, and mobile phase of 55.6% ethanol, 2.9% propanol, 40.1% methanol and 1.4% 1.05 M $NaClO_4$ at 1.0 mL/min. The applied potential for EC detection was +0.6 V. Reduced ubiquinol standards were prepared by reduction with sodium dithionite or sodium borohydride.[240]

4.2.3.2 Aqueous Antioxidants

At the time of homogenising, an aliquot of the homogenate was added to an equal volume 5% metaphosphoric acid, vortexed and microfuged (15 000 $\times g$, 4°C, 10 min) to precipitate mucosal proteins. Reduced and total ascorbate were determined as previously described.[141] Two 50 μl aliquots of the resulting supernatant were analysed for reduced and total ascorbic acid content. To the first aliquot 14.4 μl 2.58M K_2HPO_4 and to the second 14.4 μl 1% (w/v) homocysteine in 2.58M K_2HPO_4 were added. Both reactions were incubated at 37°C, 15 min and returned to ice followed by addition of 436 μl mobile phase before analysis. Samples were analysed by HPLC using a Nova-Pak C_{18}, 4 μm cartridge and mobile phase of 40 mM $NaCH_3COO$, 540 μM Na_2EDTA, 1.5 mM Q12 ion pair reagent (Regis Chemical Company, IL, USA) and 7.5% CH_3OH at 1.0 mL/min. The pH was adjusted to 4.75 with glacial acetic acid. Electrochemical detection was used with an applied voltage of +0.5 V, and samples were compared to a standard curve for quantitation.

The metaphosphoric acid supernatants of mucosal homogenates were also analysed for urate content. HPLC was performed using μBondapak C_{18}, 10 μm cartridge and mobile phase of 10 mM $NH_4H_2PO_4$, 2.5% acetonitrile, pH 4.0 at 1.2 mL/min. The applied potential for electrochemical detection was +0.76 V.[242]

4.2.4 Analyses of Thiol Status

4.2.4.1 GAPDH Activity

GAPDH activity was measured in triplicate according to Beutler.[243] Homogenates were freeze-thawed 3 times, microfuged at 15,000 $\times g$ for 10 min, 4°C. 100 μl was added to a 1 ml reaction containing, 10 mM $MgCl_2$, 0.2 mM NADH, 2 mM ATP, 5 U/ml phosphoglycerate kinase in Tris-HCl/EDTA and preincubated for approximately 10 min at 37°C. The reaction was initiated by the addition of 3-phosphoglycerate to a final concentration of 10 mM. Enzyme activity was then determined spectrophotometrically (Varian Cary 1 UV/visible spectrophotometer) by monitoring the oxidation of NADH at 37°C (molar extinction coefficient of 6.22×10^3 $M^{-1}cm^{-1}$ at 340 nm) by the reverse reaction of GAPDH coupled with phosphoglycerate kinase.

4.2.4.2 [^{14}C]-Iodoacetamide Binding

The [^{14}C]-IAM binding of mucosal proteins was performed based on the method of Brodie and Reed.[244] Mucosal homogenates were incubated with 6.6 μCi [^{14}C]-

IAM/mg protein (specific activity 58 mCi/mmol) for 30 min, and labelling was stopped by the addition of an excess of unlabelled IAM. Proteins were separated by 10% SDS polyacrylamide gel electrophoresis (SDS-PAGE) (BioRad Mini-Protean II gel system) and gels were dried onto cellulose sheets (BioRad Slab Gel Dryer 585). [^{14}C]-Methylated molecular weight markers (Amersham, UK), and rabbit muscle GAPDH (Sigma) labelled with [^{14}C]-IAM were also run on the gels. Labelled proteins and molecular weight markers were detected by autoradiography after 14 days exposure to X-ray film (Hyperfilm MP, Amersham, UK) and gels of unlabelled homogenates were stained with coomassie blue to observe corresponding protein bands.

4.2.4.3 Total Reduced Thiols

Total reduced thiols were measured using 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB).[245] Homogenates containing 0.1% sodium dodecyl sulfate (SDS) were mixed with an equal volume of 2 mM DTNB (Sigma) in 0.5 M potassium phosphate buffer pH 7.0, incubated for 15 minutes at room temperature in the dark and centrifuged to remove particulate matter. Samples were transferred to a 96 well plate and quantified using a Dynatech MR600 microplate reader. The absorbance at 410 nm of 5-thio-2-nitrobenzoic acid (TNB) released was determined (reference of 630 nm) and the thiol content calculated from a standard curve using reduced glutathione.

4.2.5 Statistical Analysis

Spectrophotometric assays were done in triplicate and all results were standardised to the protein content of the homogenates, using the BioRad micro protein assay. Data are expressed as the mean ± standard deviation, with n=9 mice per group (unless otherwise stated). Statistical differences were determined using the unpaired Student's t test.

4.3 Results

The disease course of the DSS-induced colitis in this experiment was typical of previous experiments, with development of diarrhoea followed by rectal bleeding and less weight gain compared to control mice (Appendix A). The mean MPO activity in the mucosa increased markedly from 9.2 ± 4.6 μU/g in control mice to 30.9 ± 10.1 μU/g in mice exposed to DSS for eight days ($P < 0.001$) (Figure 4.1).

The overall level of oxidant stress in the mucosa was assessed by measuring the total aqueous peroxyl radical scavenging capacity. In DSS-treated mice, the peroxyl radical scavenging capacity of the mucosa was decreased significantly to 84.3% of control mucosa ($P < 0.05$) (Figure 4.2). To discern the nature of the decrease in overall scavenging capacity, the major components of the chemical antioxidant defences were measured, revealing significant differences in the antioxidant status of the mucosa from DSS-treated mice compared to control mice.

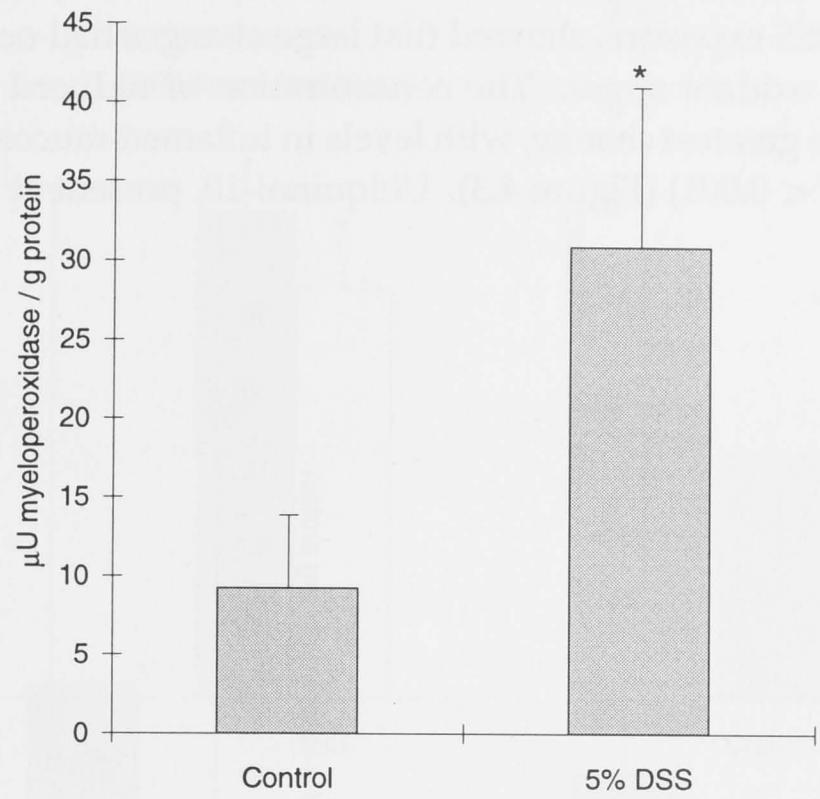


Figure 4.1: Myeloperoxidase activity in the colonic mucosa of control mice (n=9) and mice exposed to DSS for eight days (n=9). *$P < 0.001$

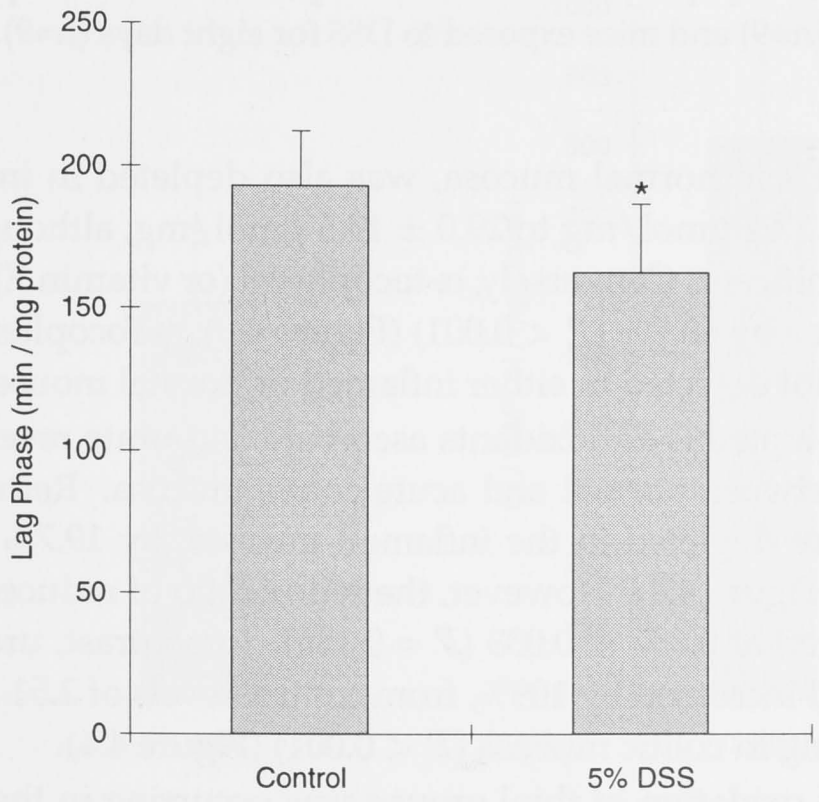


Figure 4.2: Total aqueous peroxyl radical scavenging capacity of the colonic mucosa of control mice (n=9) and mice exposed to DSS for eight days (n=7). *$P < 0.05$

The determination of three key lipid-phase antioxidants, ubiquinol-9, ubiquinol-10 (components of the mitochondrial electron transport chain) and α-tocopherol on the eighth day of DSS exposure, showed that large changes had occurred to this level of defence against oxidant stress. The concentration of reduced ubiquinol-9 in the mucosa showed the greatest change, with levels in inflamed mucosa being only 46.9% of control levels ($P < 0.001$) (Figure 4.3). Ubiquinol-10, present at less than 5% of the

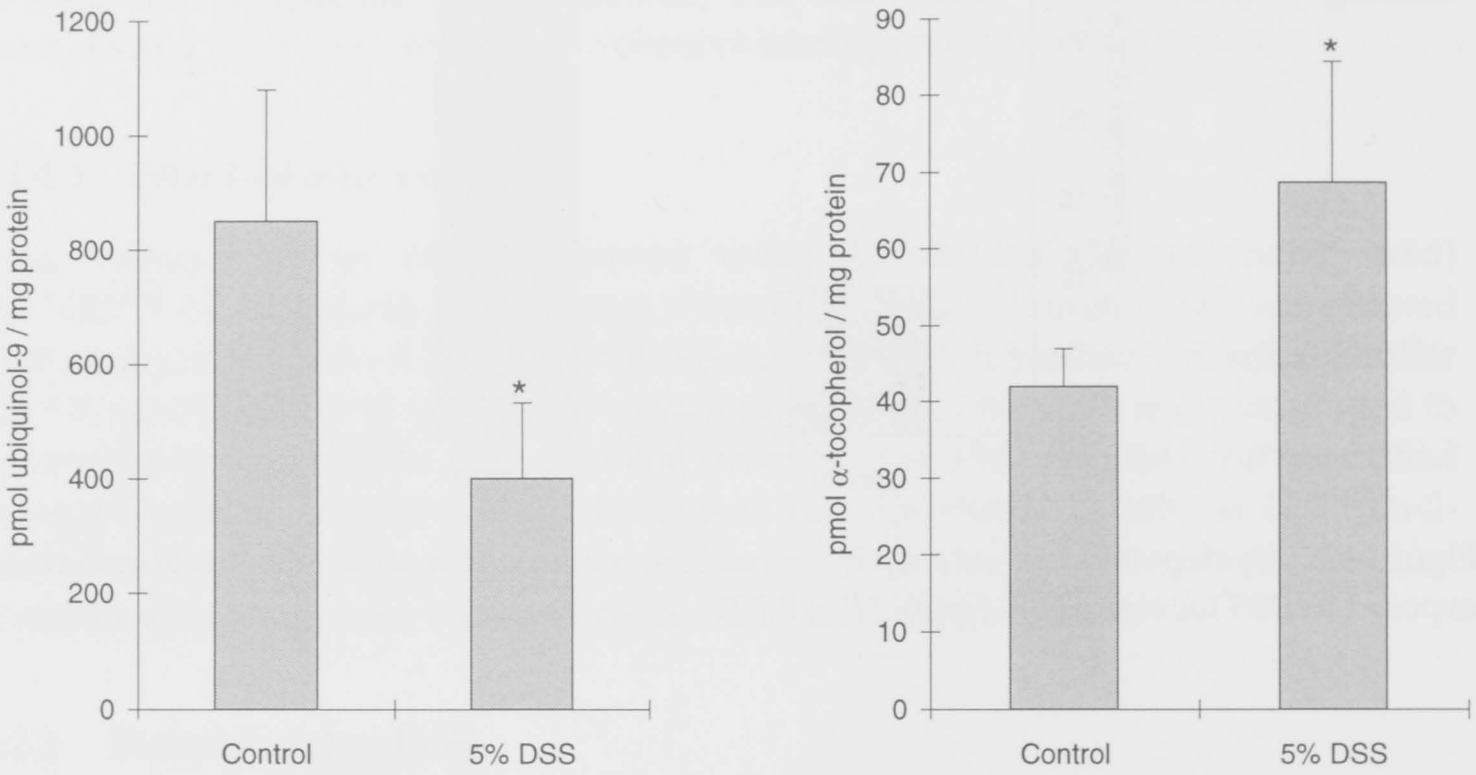


Figure 4.3: Levels of the lipophilic antioxidants, ubiquinol-9 and α-tocopherol, in colonic mucosa of control mice (n=9) and mice exposed to DSS for eight days (n=9). *$P < 0.001$

levels of ubiquinol-9 in normal mucosa, was also depleted in inflamed mucosa by 23.5%, from 38.0 ± 12.2 pmol/mg to 29.0 ± 13.5 pmol/mg, although this change was not statistically significant. Conversely, α-tocopherol (or vitamin E) levels increased in the inflamed mucosa by 63.7% ($P < 0.001$) (Figure 4.3). γ-Tocopherol, a minor isomer of vitamin E, was not detected in either inflamed or normal mouse colonic mucosa.

Analysis of the aqueous antioxidants ascorbate and urate revealed further significant differences between normal and acute colitis mucosa. Reduced ascorbate and total ascorbate were depleted in the inflamed mucosa, by 19.7% and 17.6% respectively ($P < 0.001$) (Figure 4.4). However, the redox ratio of reduced to total ascorbate remained unchanged at 0.857 ± 0.033 ($P = 0.156$). In contrast, urate levels in the inflamed mucosa had increased by 109%, from control levels of 2.51 ± 0.29 nmol/mg to 5.24 ± 1.00 nmol/mg in colitic mucosa ($P < 0.001$) (Figure 4.4).

To determine if oxidation of thiol groups was occurring in the inflamed mucosa, the level of total reduced thiols in the mucosal homogenates was measured. The total reduced thiol content of mucosa from DSS-treated mice was found to be 26.3% lower than in control mucosa ($P < 0.001$) (Figure 4.5). Mucosal GAPDH activity, identified as a direct marker of oxidant stress in IBD,[149] was also markedly decreased in the

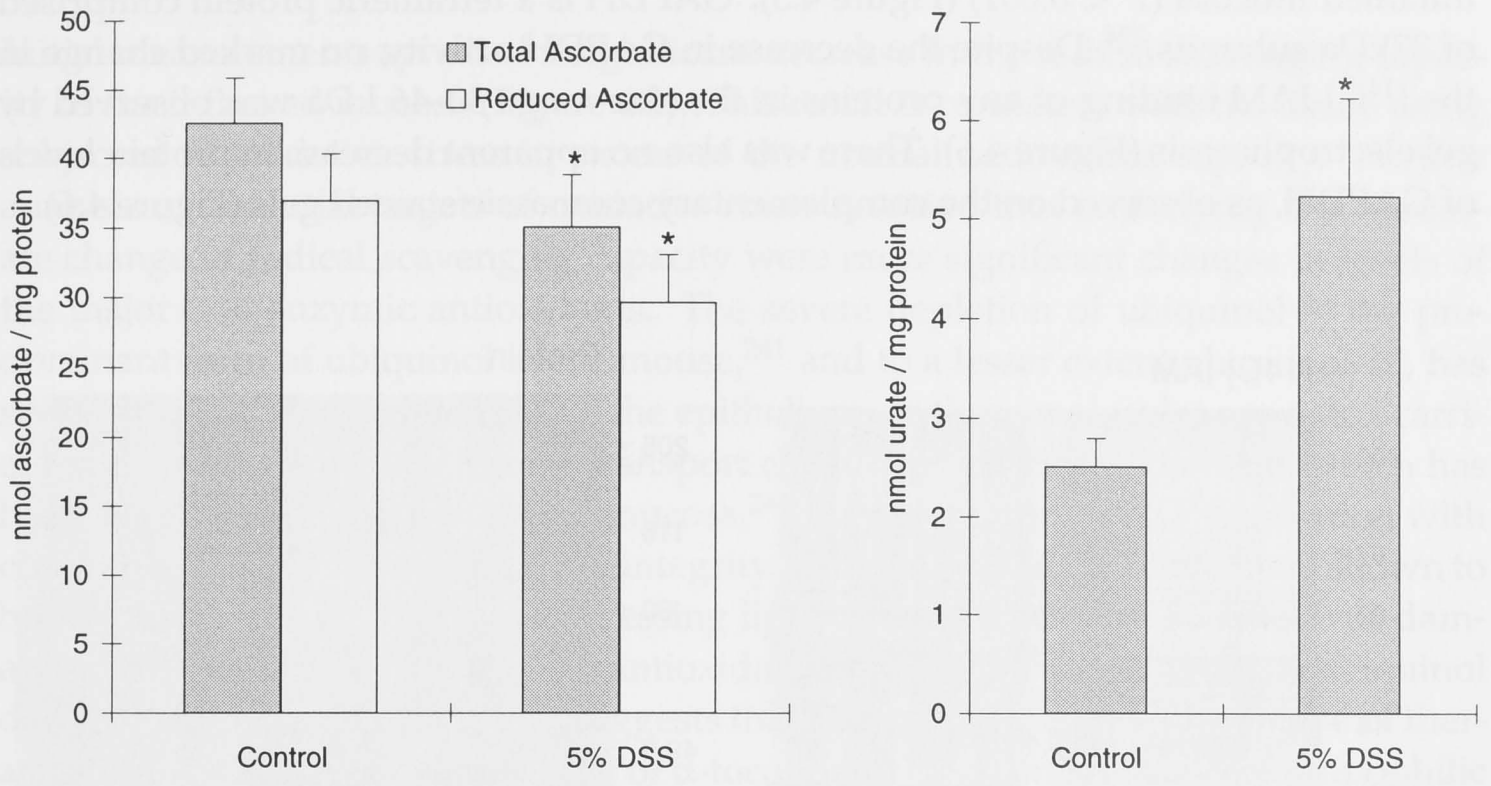


Figure 4.4: Levels of the aqueous antioxidants, ascorbate (reduced form and total of oxidised and reduced forms) and urate, in colonic mucosa from control mice (n=9) and mice exposed to DSS for eight days (n=9). *$P < 0.001$ compared to the same measurement in control mice.

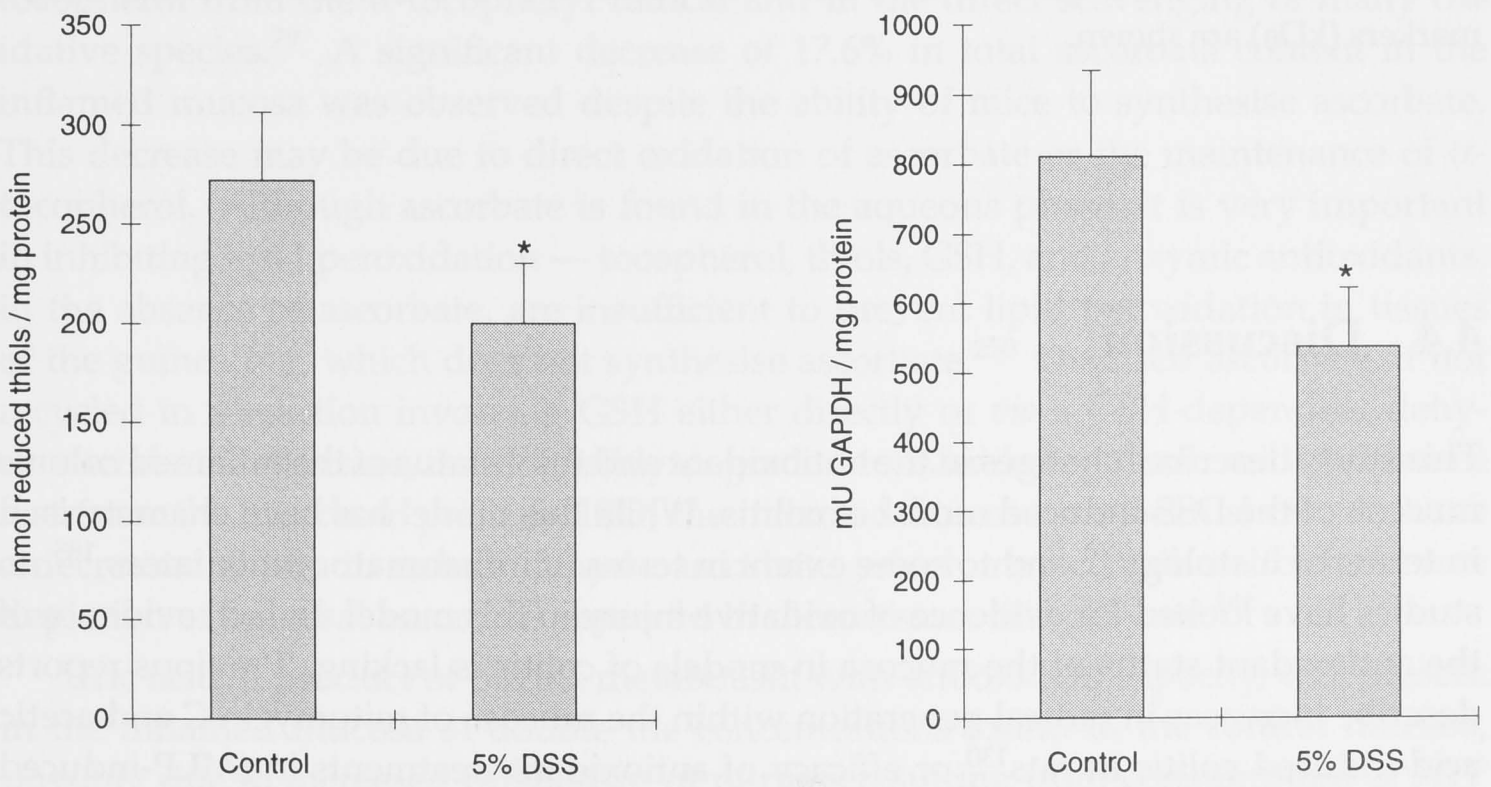


Figure 4.5: Total reduced thiol content (left) and glyceraldehyde-3-phosphate dehydrogenase activity (right) in the colonic mucosa of control mice (n=9) and mice exposed to DSS for eight days (n=9). *$P < 0.001$

inflamed mucosa by 33.8%, from 812 mU/mg in control mucosa to 538 mU/mg in inflamed mucosa ($P < 0.001$) (Figure 4.5). GAPDH is a tetrameric protein comprised of 37kDa subunits.[234] Despite the decrease in GAPDH activity, no marked change in the [^{14}C]-IAM binding of any proteins in the size range 30–46 kDa was observed by gel electrophoresis (Figure 4.6). There was also no apparent decrease in protein levels of GAPDH, as observed on the complementary coomassie stained gels (Figure 4.6).

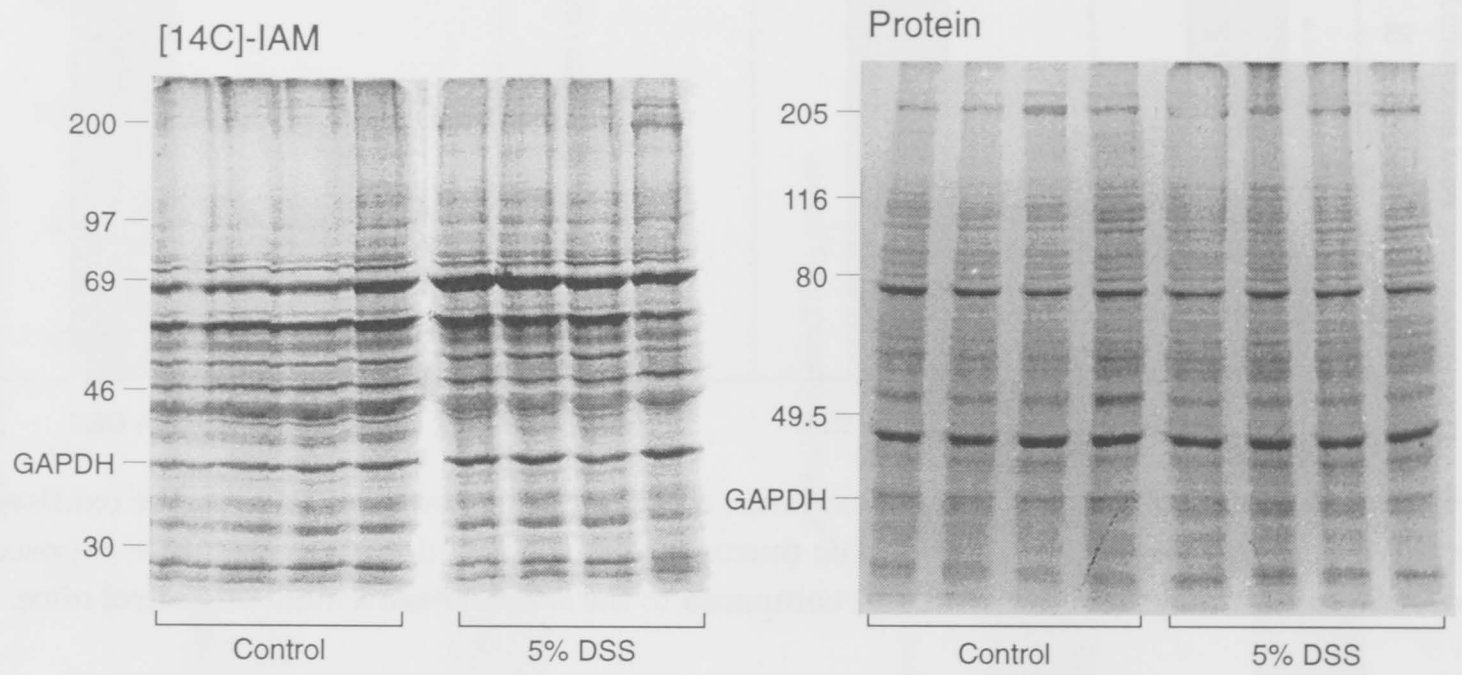


Figure 4.6: [^{14}C]-IAM labelling (left) and coomassie protein staining (right) of colonic mucosal proteins from control and DSS-treated mice. The positions of GAPDH and molecular weight markers (kDa) are shown.

4.4 Discussion

This study describes changes in the antioxidant and thiol status of the inflamed colonic mucosa of the DSS-induced model of colitis. While this model has been characterised in terms of histology,[61] and to some extent in terms of inflammatory mediators,[185] no studies have looked for evidence of oxidative injury in this model. In fact, evidence of the antioxidant status of the mucosa in models of colitis is lacking. Previous reports describe increases in radical generation within the mucosa of mitomycin C and acetic acid-induced colitis in rats[130] or efficacy of antioxidant treatments in fMLP-induced inflammation, acetic acid-induced colitis and TNBS-induced ileitis[135,168,170] but do not investigate changes in the mucosa which might reflect oxidative tissue injury. The aim of this study was to determine whether the antioxidant or thiol status of the mucosa was altered, therefore implicating oxidative injury in DSS-induced colitis, and to compare the tissue injury status to the mucosal lesion of IBD.

4.4.1 Altered Antioxidant Status

Initial measurements revealed a significant decrease in the radical scavenging capacity of the inflamed mucosa (Figure 4.2). This result is consistent with the tissue being subjected to oxidative stress in excess of the normal antioxidant capacity. Further analysis of potential targets of oxidative stress revealed that underlying this moderate change in radical scavenging capacity were more significant changes in levels of the major non-enzymic antioxidants. The severe depletion of ubiquinol-9, the predominant form of ubiquinol in the mouse,[241] and to a lesser extent ubiquinol-10, has serious potential consequences for the epithelium, as these molecules are redox carriers in the mitochondrial electron transport chain. Mitochondrial damage, which has been suggested previously in IBD mucosa,[246] may compromise ATP generation with consequent loss of cell function and integrity. Exogenous ubiquinol has been shown to be effective as an antioxidant, supressing lipid peroxidation in ischaemic liver damage[247] and increasing lipoprotein antioxidant defences.[248] The extent of ubiquinol depletion in this model of colitis suggests that ubiquinol supplements may be of therapeutic value. The mucosal content of α-tocopherol or vitamin E, the major lipophilic chain-breaking antioxidant, was greatly increased, and contrasted with the depletion of ubiquinol in the inflamed mucosa of DSS-colitis. This finding may result from the redistribution of hepatic stores of α-tocopherol, as has been suggested to occur during the oxidative stress of hyperoxia,[249] in response to the depletion of ubiquinol species and membrane antioxidant capacity.

Ascorbate is the major aqueous antioxidant, being involved in regeneration of α-tocopherol from the α-tocopheryl radical and in the direct scavenging of many oxidative species.[79] A significant decrease of 17.6% in total ascorbate content in the inflamed mucosa was observed despite the ability of mice to synthesise ascorbate. This decrease may be due to direct oxidation of ascorbate or the maintenance of α-tocopherol. Although ascorbate is found in the aqueous phase, it is very important in inhibiting lipid peroxidation — tocopherol, thiols, GSH, and enzymic antioxidants, in the absence of ascorbate, are insufficient to prevent lipid peroxidation in tissues of the guinea pig, which does not synthesise ascorbate.[250] Oxidised ascorbate, if not recycled in a reaction involving GSH either directly or *via* a GSH-dependent dehydroascorbate reductase, is irreversibly degraded to 2,3-diketogulonic acid and hence lost from the ascorbate pool. In IBD, the inflamed mucosa has been shown to have a decreased ability to reduce dehydroascorbate, which may contribute to the severe depletion of mucosal ascorbate observed in ulcerative colitis patients.[141]

Uric acid, a product of purine metabolism with antioxidant capacity, was present in the inflamed mucosa at double the concentrations found in the control mucosa, possibly due to increased catabolism of purines resulting from consumption of ATP and epithelial cell death. In acting as an antioxidant, urate is converted to allantoin.[242] Urate is also catabolised to allantoin by urate oxidase in many animals, including the mouse, although this enzyme was not detectable in the intestine of mice[251] or rats.[252] Thus allantoin levels in the mucosa may reflect the role of urate as a scavenger *in vivo*.[242] However, this potential role of urate could not be assessed, as allantoin could

not be resolved by HPLC from unidentified components of the mucosal preparation.

The alterations in the antioxidant status of the mucosa support the hypothesis that increased oxidant production is involved in DSS-induced colitis. While other pathological events, including alterations in the cellular composition of the mucosa and epithelial cell lysis, may contribute to these changes in antioxidant status in the colitis mucosa, the findings reported here are consistent with increased oxidative stress. In the case of ascorbate, the presence of the inflammatory cell infiltrate may partly conceal the significance of the changes observed, as the high ascorbate content of neutrophils and macrophages[253] may disguise its depletion by oxidation. Regardless of the cause, the alterations in antioxidant capacity leave the tissue vulnerable to oxidative tissue injury should there be an increased oxidant flux from the recruited inflammatory cells present in the inflamed mucosa.

The decreases in mucosal ubiquinol-9 and ascorbate levels, and increases in α-tocopherol and urate levels were greater in magnitude and significance than the observed decrease in total scavenging capacity of the mucosa, indicating how measurement of a single parameter, such as the total scavenging capacity of a tissue, can conceal the true extent of biochemical disturbance in a situation of potential oxidative stress. The contrasting changes of these results suggest that while some antioxidants are depleted within the mucosa, there is a compensatory increase in the levels of complementary antioxidants. This could be interpreted as evidence of a host response to maintain defences against oxidative stress.

The changes in antioxidant levels and in radical scavenging capacity of the colonic mucosa of DSS-induced colitis parallel those reported in IBD patients. Recent studies performed on inflamed and non-inflamed paired biopsies from both ulcerative colitis and Crohn's disease patients have observed decreases in ubiquinol-10 (the major form in humans[241]), total and reduced ascorbate levels, total scavenging capacity, and slight increases in α-tocopherol levels,[141,142] consistent with the changes observed in this model. However, decreased urate levels were reported, contrasting with the change seen in this model. This may reflect a difference in the intensity of the inflammation in the tissue studied — the IBD biopsies were obtained from severely inflamed mucosa, in which any excess urate may have been consumed by oxidation, whereas the mouse mucosal homogenates represent the average urate content of the whole colon. Greater depletion of other antioxidants such as ascorbate and thiol groups may be necessary before substantial consumption of urate by oxidation occurs, as is the case in plasma.[254]

4.4.2 Thiol Oxidation

This study has demonstrated alteration of the thiol status of the mucosa in DSS-induced colitis. A significant decrease in total reduced thiol content of the mucosa was found (Figure 4.5), supporting the hypothesis that oxidants may be involved in the intestinal injury observed in this model. This decrease in reduced thiols may include the depletion of low molecular weight thiols, such as GSH, and the oxidation of protein thiols, such as on GAPDH.

GAPDH, a glycolytic enzyme with a reduced thiol at its active site,[234] is very sensitive to oxidative inactivation.[103,236] Inactivation by oxidation of the active site thiol has been demonstrated by decreased [^{14}C]-IAM labelling both *in vitro*[237,238] and during carageenan-induced arthritis.[255] Recently, our laboratory has identified GAPDH as a direct marker of oxidative tissue injury in inflammatory bowel disease.[149] A decrease in the GAPDH activity in colonic epithelial cells from IBD patients correlated with a decrease in [^{14}C]-IAM binding due to the oxidation of thiol groups on the enzyme. In DSS-treated mice, a decrease in mucosal GAPDH activity was also observed when compared to control mice, which could not be accounted for by a decrease in GAPDH protein levels. There was also no change observed in [^{14}C]-IAM binding. This may be due to the sensitivity limit of this technique, as both the levels of GAPDH and the amount of inhibition measured in the mouse mucosa were much lower than those measured in human tissue. The decrease in GAPDH activity may interfere in the supply of ATP and reducing equivalents from the glycolytic pathway, although there is some debate as to the relative importance of glycolysis and fatty acid oxidation in colonocyte metabolism.[256]

Thiols have been demonstrated to be important in intestinal function. Inhibition of GSH synthesis in mice by administration of BSO, without any further toxic challenge, lead to diarrhoea and marked epithelial cell degeneration,[146,147] which was prevented by oral administration of GSH or GSH mono-ester. Luminal exposure of the rat small intestine to oxidants such as H_2O_2 and $O_2^{\bullet-}$, resulted in a 50% decrease in plasma GSH levels, while mucosal GSH levels were maintained and GSSG and protein disulfides in the mucosa increased several-fold.[257] Recently, colitis induced by intraluminal administration of the thiol blocker, iodoacetamide, has been described, further suggesting an important role for thiol compounds in maintaining mucosal integrity in the gastrointestinal tract.[148]

While the exact nature of the action of these thiol modulating agents in the intestine is not known, one candidate thiol-containing protein whose oxidation may contribute to diarrhoea is the epithelial cell Na/K-ATPase. Na/K-ATPase plays a major role in regulating electrolyte and fluid absorption. Impaired enzyme activity has been reported in IBD patients and linked to the pathogenesis of diarrhoea.[258,259] While the mechanism of inhibition of Na/K-ATPase in the inflamed mucosa is not known, inhibition by oxidants of cardiac[260] and kidney[261] Na/K ATPase activity and prevention of oxidant-mediated inhibition by the free radical scavenger vitamin E,[261] suggests that thiol oxidation in the inflamed mucosal lesion may be an important mechanism in the aetiology of diarrhoea in IBD.

Thiol oxidation may be reversible, depending on the mechanism of oxidation. Disulfides are readily reduced enzymically: GSSG by GSH reductase; and protein disulfides can be reduced by the thioredoxin system.[262,263] Thioredoxin itself undergoes reversible redox change to a disulfide group, thereby reducing the substrate protein disulfide. Thioredoxin is then restored to the reduced state by thioredoxin reductase.[262,263] Both thioredoxin reductase and GSH reductase require a supply of NADPH for reducing equivalents. The nature of the oxidation of the GAPDH thiol and whether it is reversible in IBD is unknown. Oxidation of isolated and cellular

GAPDH by peroxides is largely reversible,[235,237] but depletion of cellular GSH before oxidant exposure resulted in irreversible inactivation of the enzyme.[237] Other studies have reported irreversible inactivation of GAPDH after ozone exposure,[264,265] and after $^{\bullet}NO$ exposure, which can result in the ADP-ribosylation of the enzyme.[266–268] Recent experiments in our laboratory have demonstrated that colon epithelial cells exposed to H_2O_2 recover their GAPDH activity unassisted, but not when exposed to HOCl (McKenzie, unpublished results).

Thus, GSH and protein thiol oxidation could make important contributions to the tissue injury seen in colitis and, if reversible, may be a good target for therapeutic approaches. Preliminary success has been experienced in our laboratory with treatment of colitis with the combination of D3286 (a GSH peroxidase mimic) and lipoic acid (a thiol redox cycling compound). A reduction in the severity of the symptoms, particularly rectal bleeding, has been observed (Buffinton, unpublished results). Towards developing this possibility, further characterisation of the changes in thiol status in the inflamed mucosa of DSS colitis is required. Analysis of reduced and oxidised GSH, protein GSH conjugates, and transport of peripheral blood GSH to the mucosa during the course of the disease would provide a valuable insight into the role of thiol oxidation in the pathogenesis of colitis.

4.4.3 Conclusion

Changes were observed in antioxidant levels, total reduced thiols and GAPDH activity in the mucosa of mice with DSS-induced colitis. These observations support a role for excessive oxidant production and thiol oxidation in the tissue injury of this disease. The changes observed closely resemble those reported in human IBD tissue, indicating that DSS-induced colitis is a highly suitable model for studying further mechanisms of oxidative tissue injury in IBD.

Chapter 5

Protein Carbonyl Formation

5.1 Introduction

In DSS-induced colitis, the increased potential for oxidant production (Chapter 3), and the demonstrated depletion of antioxidant defences in the mucosa (Chapter 4) create the opportunity for oxidation and inactivation of mucosal proteins. The observation of decreased thiol content and GAPDH activity in the mucosa suggests that protein thiols may be oxidised in DSS-induced colitis (Chapter 4). The measurement of protein carbonyls is an alternative indicator of protein oxidation which is well established *in vitro* and to a lesser extent *in vivo*.[105] Investigation of protein carbonyls in colitis may provide further evidence of protein oxidation and potentially identify specific proteins which are susceptible to oxidation and consequent malfunction.

Carbonyls are non-specific chemical groups comprising ketones and aldehydes (Figure 5.1). While a natural part of many biochemicals, in particular nucleic acids, they can also be formed on proteins as a result of oxidation. The f ormation of protein carbonyls has been studied after metal-catalysed oxidation (MCO) of proteins and oxidation by HOCl.[269,270] More recently, aldehydes in cigarette smoke have been shown to produce carbonyls on plasma proteins,[271,272] and lipid peroxidation was shown to lead to protein carbonyl formation in an indirect manner.[273] With this range of mechanisms, many amino acids are susceptible to carbonyl oxidation including lysine, arginine, proline and histidine.

Increases in protein carbonyl levels have been reported in several diseases where RONS are implicated, such as aging,[274] ischemia-reperfusion,[275,276] and amyotrophic lateral sclerosis.[277] In inflammation, higher carbonyl content has been measured in the synovial fluid of rheumatoid arthritis patients compared to osteoarthritis.[278] While oxidants have been implicated in the tissue injury of IBD, only one report of direct evidence of protein oxidation, as GAPDH thiol oxidation, in the inflamed tissue has been made.[149] The possibility of iron-mediated oxidation in colitis (Chapter 1.3.1)[137] and the potential for excessive HOCl production (Chapter 3) provide support for an investigation of protein carbonyls in DSS-induced colitis and IBD.

Carbonyl groups are detected by derivatisation with dinitrophenylhydrazine (DNPH) to form a stable hydrazone (Figure 5.1).[105] Dinitrophenyl (DNP) is both a chromaphore and a hapten. Total carbonyls can be determined after derivatisa-

tion by spectrophotometric measurement of the hydrazone product at 360–390 nm.[105] Carbonyl-containing proteins can also be detected using anti-DNP antibodies by western blotting, a technique described recently which allows identification of individual oxidised proteins in whole tissue.[279] Sample proteins are derivatised with 2,4-dinitrophenyl hydrazine, separated by SDS-PAGE and then analysed for carbonyl content by immunoassay with anti-DNP antibodies. This approach has been used in a semi-quantitative manner to show that in plasma, fibrinogen is twenty times more susceptible to oxidation in an iron/ascorbate system than is the abundant plasma protein, albumin.[279]

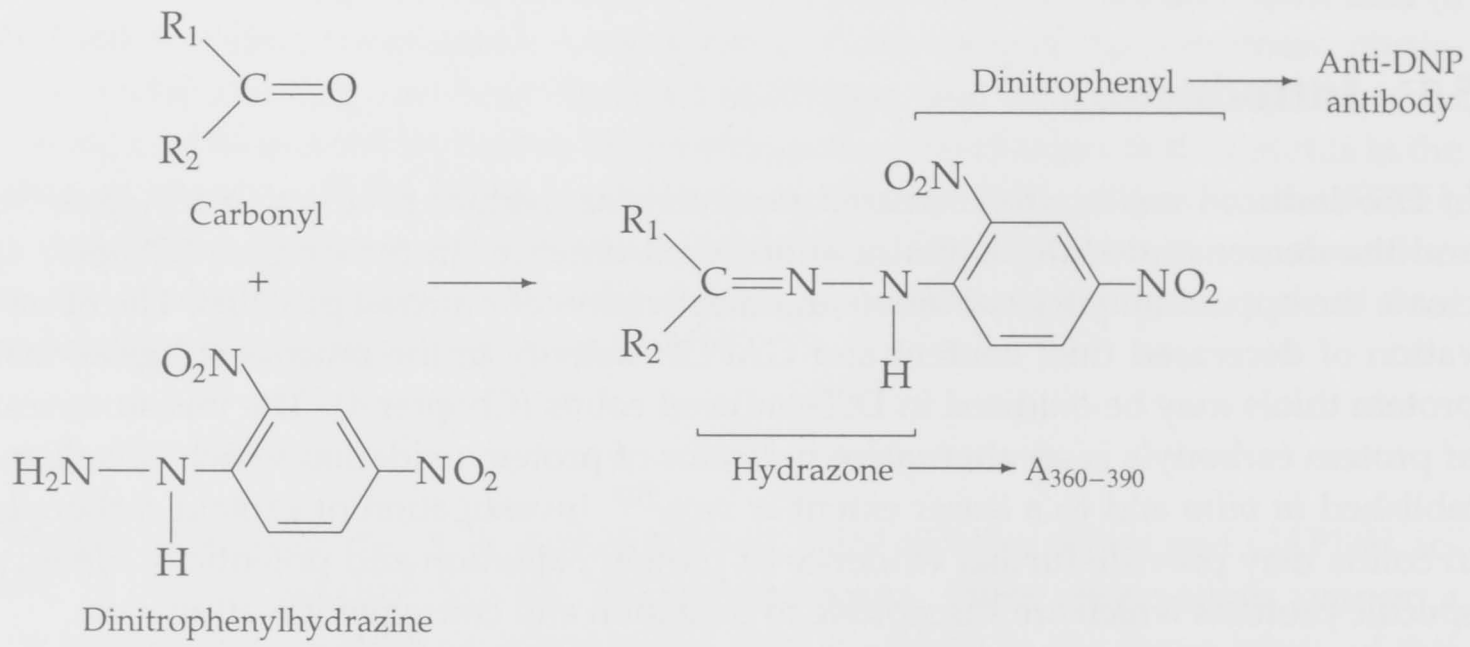


Figure 5.1: Reaction of carbonyl groups with DNP results in the production of a hydrozone. The hydrazone can then be quantified by measuring absorbance at 350–390 nm, or the DNP group can be detected using anti-DNP antibodies.

The oxidation of proteins by MCO has been studied in considerable detail *in vitro*, and several enzymic or nonenzymic systems can be used to mediate metal-catalysed protein oxidation.[108] One nonenzymic system is iron/ascorbate, where iron is supplied to bind to proteins and ascorbate reduces the iron, enabling it to participate in $^{\bullet}OH$ generation in the presence of O_2. It has been used in many situations including oxidation of glutamine synthetase,[280] plasma proteins,[279] and can also mediate oxidative damage to nucleic acids and lipids.[108]

In this chapter, the formation of protein carbonyls in the colonic mucosa was examined, by both spectrophotometry and by western blotting. Mouse mucosal homogenates were oxidised *in vitro* using iron/ascorbate, H_2O_2, HOCl and $^{\bullet}NO$ to determine which oxidants may be responsible for carbonyl formation and to identify possible susceptible proteins. These approaches were then applied to mucosa from control mice and mice with acute DSS-induced colitis, and mucosal biopsies from IBD patients, to determine whether carbonyl protein oxidation may play a role in the mucosal tissue injury of colitis.

5.2 Methods

5.2.1 *In vitro* Oxidation of Mucosal Homogenates

Mucosa was collected from healthy male 7 week old CBA/H mice and frozen on dry ice. Mucosa was homogenised at 50 mg/ml, and the homogenates form 3–6 mice pooled to provide sufficient material for each experiment. Triplicate aliquots of homogenate (diluted to 20 mg tissue/ml, approximately 1 mg protein/ml) were then exposed to oxidants at 37°C in PBS containing 25 mM Hepes, pH 7.2, and 1 mM phenylmethylsulfonyl fluoride (PMSF) to inhibit protein degradation during exposure to oxidants.

Homogenates were oxidised by the iron/ascorbate method as used by Shacter *et al.*[279] 20 mM ascorbate and 20–500 μM $FeSO_4$ were added to the incubation buffer (pH adjusted to 7.2 after addition of ascorbate and iron) and samples were incubated for 5 hr. Homogenates were also exposed to H_2O_2, HOCl and diethylamine NONOate, a $^{\bullet}NO$ donor, at concentrations from 0.1–10 mM for 30 min (see Section 3.2.4 for details of preparation of oxidants). At the end of the incubation time, samples were cooled on ice, centrifuged at 15,000×*g*, 4°C, 5 min, then aliquotted, frozen on dry ice and stored at -70° C for later analysis of carbonyl groups and total reduced thiols.

5.2.2 Colitis

Colitis was induced in 6–7 week old male CBA/H mice by administering distilled water supplemented with 5% DSS *ad libitum* for 7–11 days (Section 2.2.1). Observation of mice and collection of descending and transverse colonic mucosa was performed as described previously (Section 2.2.2) and the disease course details can be seen in Appendix A.

Mucosal homogenate supernatants were analysed spectrophotometrically for carbonyl and total reduced thiol content. Protein carbonyls were also investigated by western blotting analysis.

5.2.3 Human Biopsy Collection

Mucosal tissue specimens were obtained by biopsy of paired histologically normal (non-inflamed) and inflamed sites in patients with inflammatory bowel disease undergoing colonoscopy. Diagnosis of ulcerative colitis or Crohn's disease was confirmed by hospital histopathology on tissue samples taken adjacent to those used for experimental analysis. Biopsy samples were snap frozen on dry ice and stored at -70° C. Protocols for obtaining tissue samples used in this study were in accordance with protocols approved by the Ethics Committees from both ACT Health Institutions Committee and JCSMR.

Nine pairs of inflamed and non-inflamed biopsies from patients with active CD (n=2) or UC (n=7) were examined. Four patients with UC were receiving both steroid and aminosalicylate therapy, one CD patient was receiving steroids and azothioprine,

and the remaining 4 patients were receiving no drug treatment. Patient ages ranged from 22–59 years.

As the amount of clinical material obtained from biopsies was insufficient to perform total carbonyl analysis, western blotting analysis for protein carbonyls was performed. To assess objectively the relative intensity of the anti-DNP signal from inflamed and non-inflamed tissue, the films and blots stained for protein were digitized, the darkness of each lane area quantified and background subtracted using the program NIH Image. The ratio of the signal in inflamed to non-inflamed mucosa, after correcting for differences in protein staining, was calculated for 9 biopsy pairs, and categorised as greater in control mucosa (< 0.95), unchanged (0.95–1.05) or greater in inflamed mucosa (>1.05).

5.2.4 Spectrophotometric Determination of Carbonyl Groups

Carbonyl groups were determined according to the method of Levine *et al.*[105] Homogenates were centrifuged 15,000$\times g$ 5 min to remove debris and divided into 4 aliquots of 200 μl (approximately 0.2 mg protein). Proteins were precipitated by the addition of 100 μl 20% TCA for 5 min on ice, and centrifuged 4,000$\times g$, 5 min. The pellet was redissolved in 100 μl 0.2 M NaOH, and 100 μl of 2 M HCl or 10 mM DNPH (Fluka, Switzerland) in 2 M HCl added to duplicate aliquots for sample blanks or the derivatising of carbonyl groups respectively. Samples were reacted for 30 min at room temperature. Proteins were re-precipitated with TCA, and washed three times with 500 μl 1:1 ethanol:ethyl acetate, with 15 min stands to remove excess DNPH. Samples were redissolved in 200 μl 6M guanidine buffer containing 20 mM KH_2PO_4, pH 2.3 and the absorbance read at 370 nm in micro quartz cuvettes requiring a volume of 75 μl (Hellma, Germany) in a Cary 1 UV/visible spectrophotometer. The carbonyl content in nmol/mg protein was calculated using a molar extinction coefficient of 22,000 $M^{-1}cm^{-1}$ at 370 nm[105] after subtraction of the blank absorbance. Protein concentration of the homogenates was determined by the BioRad protein assay on an aliquot of homogenate taken before derivitisation. While some protein was lost in the wash steps, the loss was consistent across control and inflamed samples (data not shown).

Data are expressed as mean $\pm$ standard deviation, and statistical differences were determined using the unpaired Student's t test, with significance considered as $P < 0.05$.

5.2.4.1 Assessment of DNA Content of Samples

DNA is a common contaminant influencing the measurement of protein carbonyls. Tests were performed in an attempt to remove interfering DNA and therefore reduce the background carbonyl content of the samples. DNA was removed by precipitation with 1% streptomycin sulfate. To nine volumes of mucosal homogenate supernatant, one volume of 10% streptomycin sulfate in 50 mM hepes was added. Samples were let stand on ice for 15 mins before being centrifuged at 15,000$\times g$, 4°C, 10 min.

To monitor DNA content, the ratio of absorbance 280nm/260nm was determined for representative samples of control and inflamed tissue before and after the addition of streptomycin sulfate.[105] In an alternative approach, normal mucosal homogenate supernatants (100 μl) containing 2 mM PMSF were incubated with 50–200 units DNase II (Calbiochem) for 15–120 min, and the carbonyl content determined.

For comparison of control mice and mice exposed to 5% DSS, carbonyl content was determined with and without streptomycin sulfate precipitation. Streptomycin sulfate precipitation was performed as above, and the supernatant collected and analysed by the carbonyl assay above. To confirm the successful removal of DNA by this method for these samples, comparisons of the DNA and protein content of the supernatants was performed on two control and DSS-exposed samples before and after streptomycin sulfate precipitation. As streptomycin sulfate is known to interfere in the BioRad protein assay, and may interfere in the DNA assay, aliquots of supernatant (treated and untreated) were precipitated with TCA, and washed with 4% TCA to remove residual streptomycin sulfate, before being redissolved in NaOH, neutralised, diluted in appropriate buffers and used in the assays as described previously (Section 2.2.3).

Carbonyl assays on *in vitro* oxidised homogenates were performed without DNA removal. To confirm that oxidant exposure of the mucosa had not altered the amount of DNA present in the supernatant, the DNA concentration of the supernatants was determined using the Hoechst assay described in Chapter 2, without the freezing and thawing procedure performed when assaying total DNA.

5.2.5 Western Blotting for Carbonyl Groups

Immunoblot analysis for protein carbonyls was performed according to Shacter *et al.*[279] 50 μl homogenate supernatant was precipitated with 25 μl 20% TCA on ice 5 min and centrifuged 4,000$\times g$. The pellet was dissolved in 15 μl 6% sodium dodecyl sulfate (SDS) and mixed with an equal volume of 10 mM DNPH in 10% trifluoroacetic acid. Derivatisation was allowed for 15–30 min at room temperature, when 20 μl sample buffer (2 M Tris buffer containing 30% glycerol and 1 M β-mercaptoethanol (BioRad)) was added. 10 μg protein was loaded per well as determined by the BioRad protein assay. A negative control, of mucosal proteins reacted with 10% trifluoroacetic acid only, was also loaded.

Samples were subjected to 9% SDS-PAGE (BioRad Mini-Protean II gel system) followed by electroblotting (BioRad Mini Trans-blott transfer cell) onto nitrocellulose (Hybond, Amersham). Replicate blots were stained for protein with amido black or were assayed immunologically for the presence of DNP-conjugated proteins. Blots were blocked with gelatin and incubated with polyclonal rabbit anti-DNP antibody (Sigma, diluted 1/2000) followed by a horse radish peroxidase-conjugated sheep anti-rabbit antibody (Silenus, diluted 1/10,000). Bands containing DNP proteins were visualised by enhanced chemiluminescence (Amersham ECL kit) and exposure of film (Hyperfilm-ECL, Amersham).

5.2.6 Protein Sequence Analysis

Freshly homogenised mouse colonic mucosa was subject to SDS-PAGE as described above and stained for protein with coomassie blue. The 48 kDa band was excised from the gel, the protein eluted out of the gel, adsorbed onto polyvinyl difluoride membrane, and the NH_2-terminal amino acid sequence was determined using ABI Procise 494 automated peptide sequencer (Applied Biosystems, Inc). Sequence analysis was performed by the Biomolecular Resource Facility of JCSMR.

5.2.7 Total Reduced Thiols

The total reduced thiol content of the mucosal homogenate supernatants after *in vitro* exposure to oxidants was determined as described previously (Section 4.2.4.3). However, this method could not be applied to the samples containing iron and ascorbate, as iron/ascorbate can hydrolyse the 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) to release the chromophore, thus giving artificially high results.

5.3 Results

5.3.1 Assessment of DNA Removal

To assess if DNA removal with streptomycin was necessary in mucosal homogenate supernatants, the ratio of absorbance 280nm/260nm was determined of representative samples of control and inflamed tissue before and after the addition of 1% streptomycin sulfate. This step is considered necessary to remove interfering nucleic acids if the ratio 280/260 is less than 1.[105] While the ratio 280/260 was below 1 for mucosal homogenate supernatants, no difference was evident between mucosa from control and DSS-exposed mice (Table 5.1). A streptomycin sulfate precipitation step did not increase the value of the 280/260 ratio. Further attempts were made to remove pos-

Sample	Before streptomycin	After precip.
Control	0.620	0.606
Control	0.648	0.624
DSS	0.672	0.650
DSS	0.680	0.695

Table 5.1: Ratio of absorbance 280nm/260nm of mucosal homogenate supernatants from two control and two DSS-exposed mice before and after a precipitation step using 1% streptomycin sulfate.

sible contaminating nucleic acids by incubation of control homogenate supernatants with DNase, but no decrease in nmol carbonyl/mg protein was achieved. Thus it appeared that the removal of nucleic acids was not necessary for the determination of

protein carbonyls in mucosal homogenates and carbonyl assays were performed on untreated mucosal homogenate supernatants.

5.3.2 *In vitro* Oxidation of Mucosal Proteins

5.3.2.1 Total Carbonyl Analysis

To examine which oxidants may be involved in carbonyl formation on mucosal proteins, normal mouse colonic mucosal homogenates were exposed to an iron/ascorbate oxidation system, and the neutrophilic oxidants H_2O_2, HOCl and $^{\bullet}NO$ *in vitro*. Iron/-ascorbate oxidation of homogenates resulted in increased carbonyl content, with exposure to 20 μM iron/20 mM ascorbate for 5 hr producing an 80% increase in carbonyl groups in the mucosal homogenate supernatant. Carbonyl formation increased with increasing concentrations of iron, with 6.7±0.1, 7.4±0.8, and 10.3±0.8 nmol carbonyl/mg protein being measured after exposure to 20, 100 and 500 μM iron respectively (control levels 3.7 ±0.4 nmol/mg protein) (Figure 5.2).

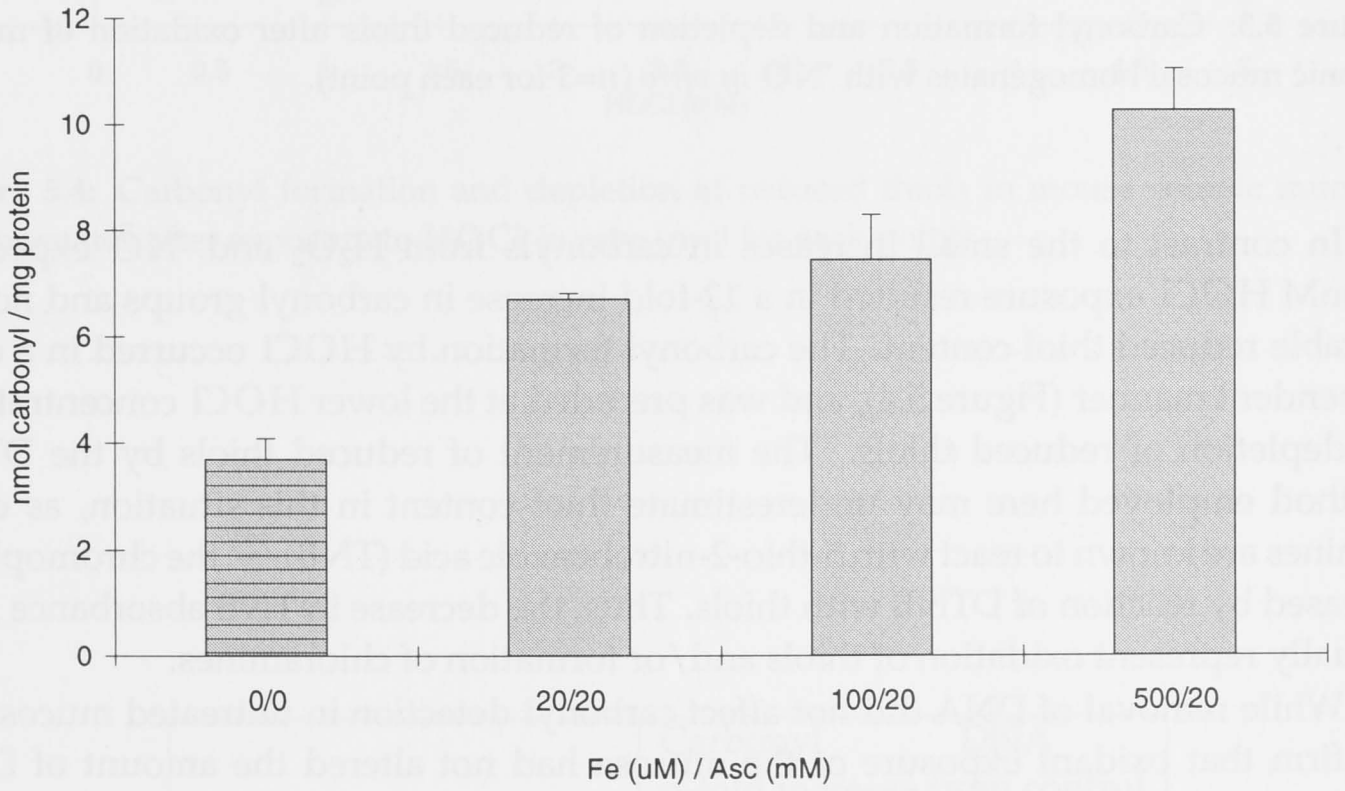


Figure 5.2: Carbonyl formation after oxidation of mouse colonic mucosal homogenates with the iron/ascorbate system (n=3 for each point).

Exposure of mucosal homogenates to 10 mM H_2O_2 did not result in protein carbonyl formation (4.5±1.4 and 3.9±0.7 nmol/mg protein with 0 and 10 mM H_2O_2 respectively), although it did result in a 25% decrease in reduced thiol content, from 113.8±6.6 to 85.9±7.2 nmol/mg protein. Exposure of homogenates to 10 mM $^{\bullet}NO$ resulted in a 70% increase in protein carbonyl groups (from 3.3±0.5 to 5.6±1.7 nmol/mg protein), but no increase was observed at the lower concentrations of $^{\bullet}NO$. Exposure of homogenates to 1 and 10 mM $^{\bullet}NO$ also resulted in depletion of reduced thiols by 42% and 90% respectively (Figure 5.3).

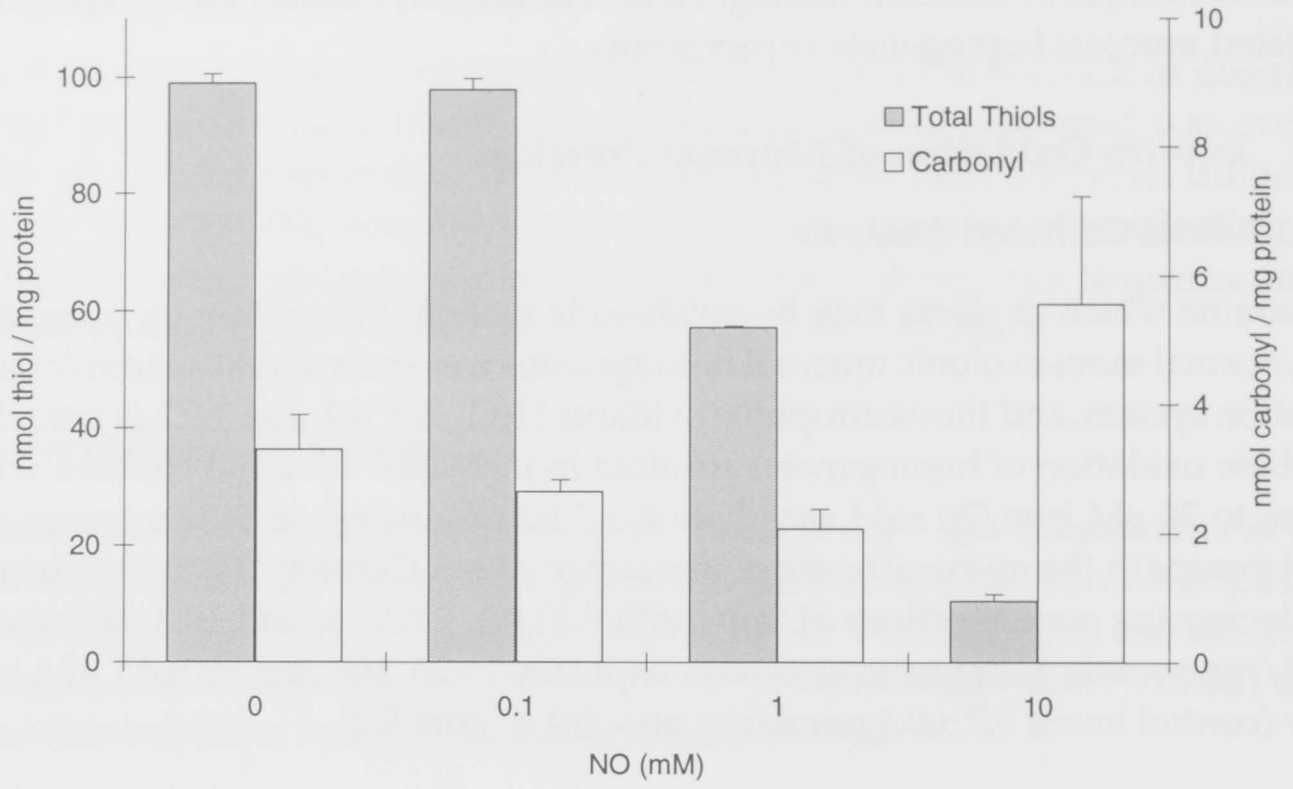


Figure 5.3: Carbonyl formation and depletion of reduced thiols after oxidation of mouse colonic mucosal homogenates with ˙NO *in vitro* (n=3 for each point).

In contrast to the small increases in carbonyls from H_2O_2 and ˙NO exposure, 10 mM HOCl exposure resulted in a 12-fold increase in carbonyl groups and no detectable reduced thiol content. The carbonyl formation by HOCl occurred in a dose dependent manner (Figure 5.4), and was preceded at the lower HOCl concentrations by depletion of reduced thiols. The measurement of reduced thiols by the DTNB method employed here may underestimate thiol content in this situation, as chloramines are known to react with 5-thio-2-nitrobenzoic acid (TNB),[229] the chromophore released by reaction of DTNB with thiols. Thus, the decrease in TNB absorbance may actually represent oxidation of thiols and/or formation of chloramines.

While removal of DNA did not affect carbonyl detection in untreated mucosa, to confirm that oxidant exposure of the mucosa had not altered the amount of DNA present in the supernatant, the DNA content of the homogenate supernatant after oxidation was measured. Changes in the DNA content were observed, but they were too small to account for the increase in carbonyl content observed (Table 5.2).

5.3.2.2 Anti-DNP Analysis

The colonic mucosal homogenates exposed oxidants *in vitro* were also analysed by western blotting analysis for carbonyl groups. This method was established recently and has a detection limit estimated at 1 pmol of protein-associated carbonyls, with as little as 2 μg protein being required[279] compared to the minimun of 400 μg protein required in the spectrophotometric method. Application of the immunodetection technique to plasma oxidised by iron/ascorbate revealed that fibrinogen was approx-

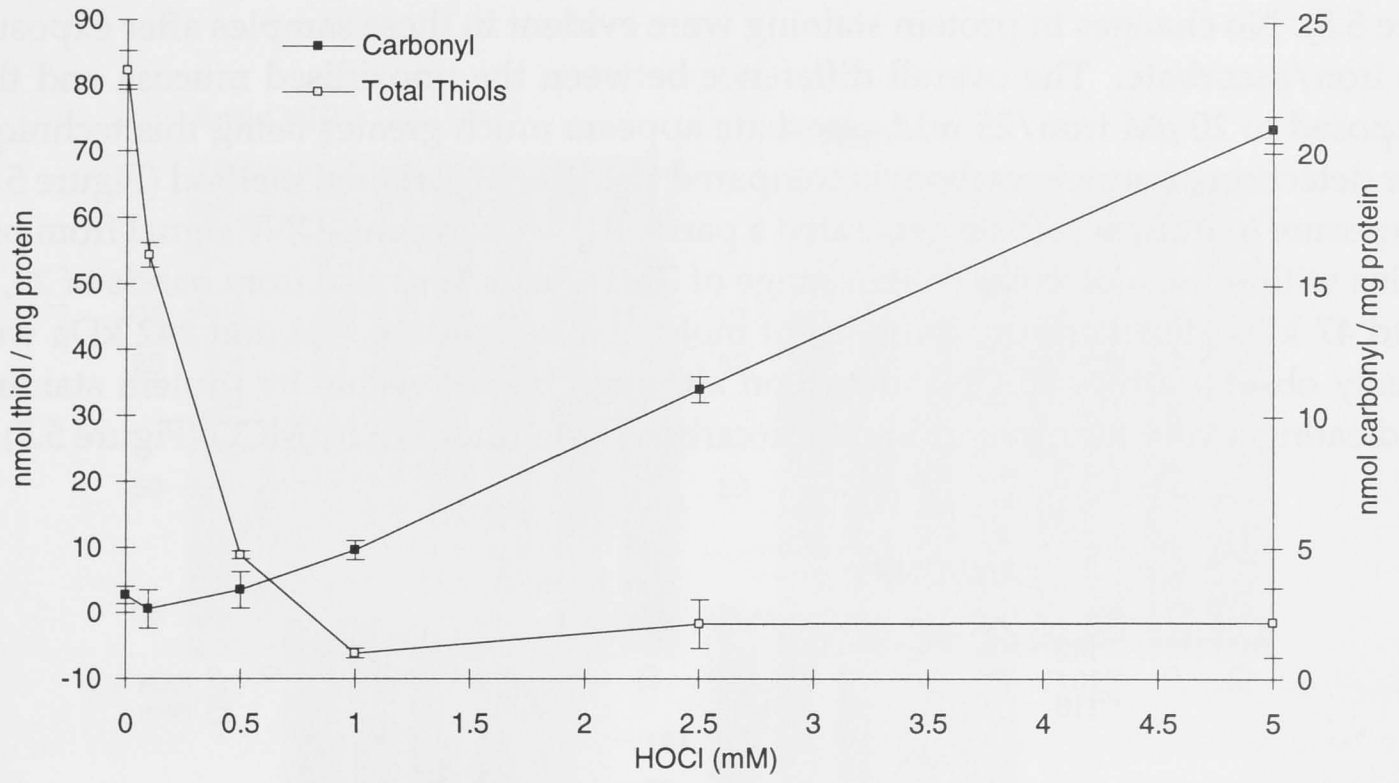


Figure 5.4: Carbonyl formation and depletion of reduced thiols in mouse colonic mucosal homogenates after exposure to HOCl *in vitro* (n=3 for each point).

		Carbonyl	DNA
		n-fold increase from control	
iron/ascorbate	20/20	0.81	-0.05
(μM/mM)	500/20	1.78	0.65
$^{\bullet}NO$ (mM)	10	0.70	0.48
HOCl (mM)	2.5	2.5	0.53
	10	12.2	1.47

Table 5.2: Increase in DNA content of homogenate supernatants after oxidant exposure. This table has been constructed from the data displayed in Figures 5.2, 5.3 and 5.4.

imately 20 times more susceptible to oxidation than albumin.[279]

Normal mouse colonic mucosa exposed to iron/ascorbate displayed many protein bands which had increased carbonyl content compared to the unoxidised mucosal homogenate, evident as a darkening of bands observed by anti-DNP detection (Figure 5.5). No changes in protein staining were evident in these samples after exposure to iron/ascorbate. The overall difference between the unoxidised mucosa and that exposed to 20 μM iron/25 mM ascorbate appears much greater using this technique for detection of protein carbonyls compared to the total carbonyl method (Figure 5.2). Exposure to iron/ascorbate generated a particularly strong anti-DNP signal from proteins within the molecular weight range of 75–110 kDa, and also from bands of 29, 35 and 47 kDa. Furthermore, proteins of molecular weight 116, 131 and 142 kDa were easily observed by anti-DNP detection although barely visible by protein staining, indicating a very high susceptibility to carbonyl modification by MCO (Figure 5.5).

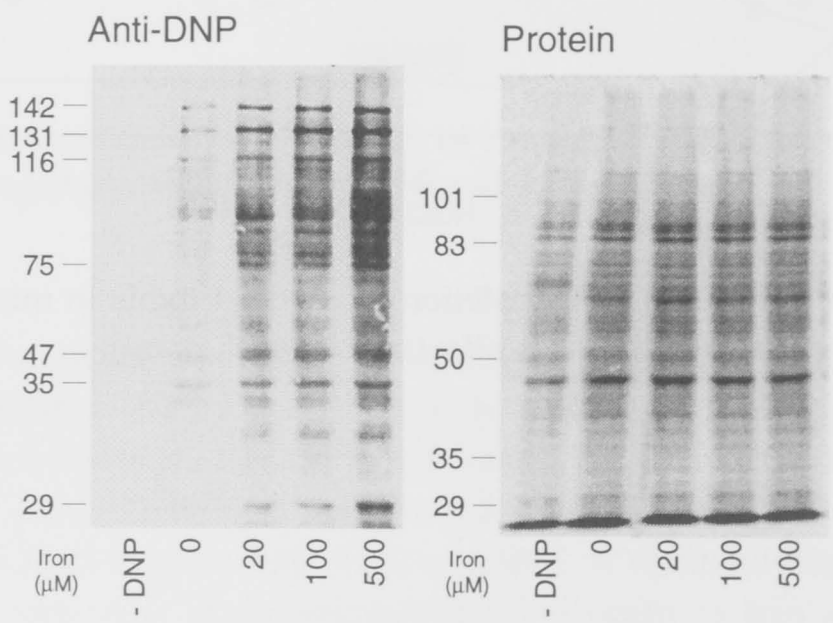


Figure 5.5: Oxidation of mouse colonic mucosal proteins by iron/ascorbate: detection of carbonyl groups by immunoblotting with anti-DNP antibody (left) and corresponding staining for protein with amido black (right). Molecular weights (kDa) of some carbonyl modified proteins (left) or molecular weight markers (right) are indicated. -DNP lane contains underivatised proteins for negative control.

Western blotting analysis of mucosa exposed to HOCl showed that oxidation by low concentrations of HOCl, 0.1 and 0.5 mM, generated carbonyl groups on many different proteins (Figure 5.6) particularly in the 45–100 kDa molecular weight region, while no changes in the protein staining were observed. At these concentrations of HOCl, no significant increase in total carbonyl content was detected spectrophotometrically (Figure 5.4). At 0.5 mM HOCl, high molecular weight material with strong anti-DNP signal appeared which was not visible in the protein staining, suggesting it was rich in carbonyl groups. At higher HOCl concentrations (1–10 mM), protein aggregation or crosslinking was evident by protein staining, and was accompanied by fragmentation of proteins with smearing both of protein staining and particularly of

anti-DNP signal. At these concentrations of HOCl, aggregation and crosslinking of proteins was so great that some material did not penetrate the 4% polyacrylamide stacking gel (not shown).

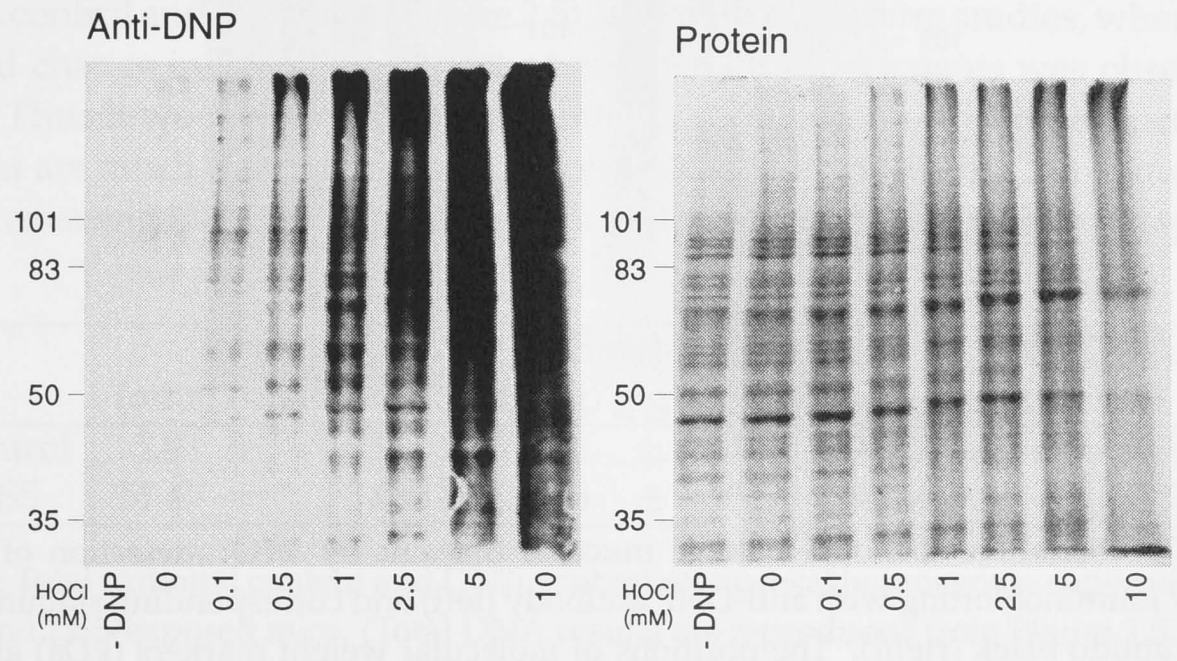


Figure 5.6: Oxidation of mouse colonic mucosal proteins by HOCl: detection of carbonyl groups by immunoblotting with anti-DNP antibody (left) and corresponding staining for protein with amido black (right). The positions of molecular weight markers (kDa) are shown. -DNP lane contains underivatised proteins for negative control.

Despite the high sensitivity of this technique, western blotting of homogenates exposed to 0.1 or 1.0 mM $^{\bullet}NO$ did not reveal carbonyl formation. The increased carbonyl content after exposure to 10 mM $^{\bullet}NO$ was visible as general darkening of protein bands, with no change in the pattern of protein staining (Figure 5.7). Minimal carbonyl formation was observed in the homogenates exposed to H_2O_2 (Figure 5.8).

While the concentration of oxidants used in these studies may not be expected to occur throughout the whole tissue, they are not unreasonable in terms of the immediate vicinity of activated neutrophils or macrophages. Superoxide concentrations generated around activated neutrophils have been estimated at 5 mM,[137] and similar concentrations of H_2O_2 and HOCl may potentially be achieved.[28,71] If concentrated within a phagosome or neutrophil-substrate adhesion cleft, oxidant concentrations may reach several-fold higher concentrations than this figure.[137] Babbs has calculated that luminal iron concentrations may be as high as 320μM in the human colon.[159] The oxidant concentrations used in this study result in comparable oxidant:target protein ratio as used by other investigators in studies on oxidant toxicity[75,149,236] and, excluding 5 and 10 mM HOCl, induced increases in carbonyl groups comparable with those reported in other disease situations (Section 5.4.2).

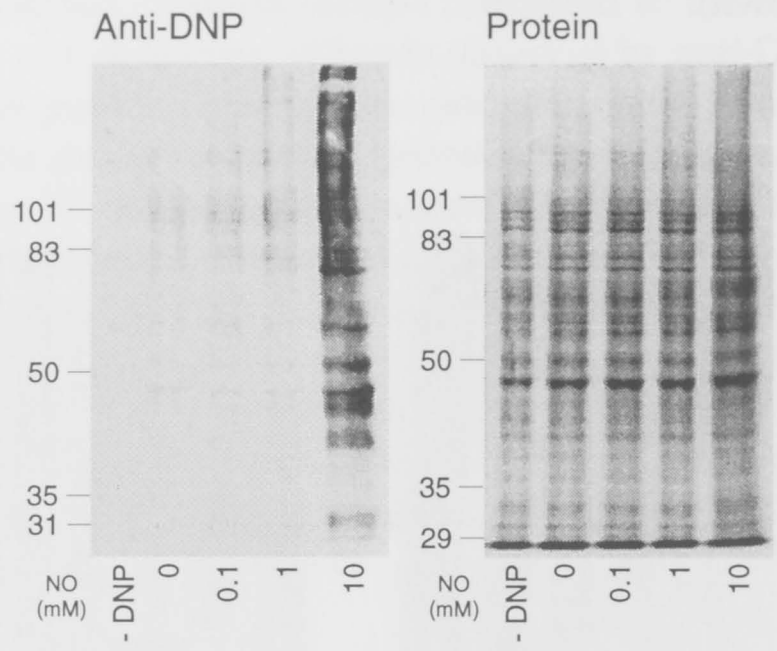


Figure 5.7: Oxidation of mouse colonic mucosal proteins by $^{\bullet}NO$: detection of carbonyl groups by immunoblotting with anti-DNP antibody (left) and corresponding staining for protein with amido black (right). The positions of molecular weight markers (kDa) are shown. -DNP lane contains underivatised proteins for negative control.

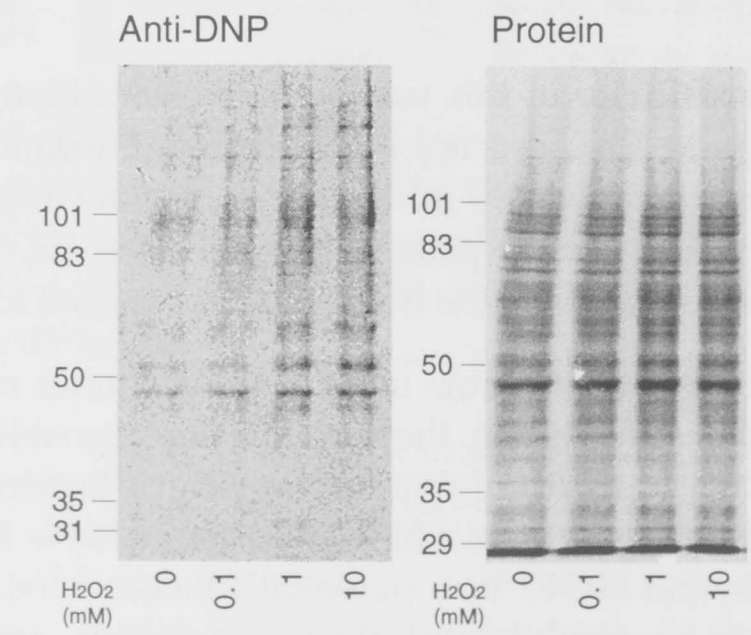


Figure 5.8: Oxidation of mouse colonic mucosal proteins by H_2O_2: detection of carbonyl groups by immunoblotting with anti-DNP antibody (left) and corresponding staining for protein with amido black (right). The positions of molecular weight markers (kDa) are shown.

5.3.3 Protein Carbonyl in DSS Colitis

In inflamed mucosa from DSS-induced colitis in mice, initial measurements showed a 4-fold increase in carbonyl content of the mucosa (Table 5.3). However, this was accompanied by a 10-fold increase in the DNA content of the supernatant. This contrasted with the total DNA content of the whole homogenates which was unchanged between control and DSS mice (Figure 2.5), and with the *in vitro* studies, where at most a 1.5-fold change in DNA levels in the homogenate supernatants was observed (Table 5.2). Thus it would seem that in the mucosa from mice with DSS-induced colitis, the nuclei are much more fragile and susceptible to rupture during the homogenising process, releasing DNA into the supernatant. To overcome this problem, streptomycin

	Carbonyl (nmol/mg prot)	n	Soluble DNA (μg/mg prot)	n	Total DNA (μg/mg prot)	n
Control	5.9 ± 0.9	10	8.8 ± 1.5	10	175.2 ± 50.9	9
DSS	24.4 ± 3.0	10	70.8 ± 15.5	9	169.3 ± 17.7	9

Table 5.3: Total carbonyl content and DNA content of mucosal homogenate supernatants from normal and DSS-exposed mice. (Total DNA results are reproduced from Figure 2.5.)

sulfate was used to remove the DNA from the samples. This also resulted in the apparent loss of 15–20% of the protein from the samples (Table 5.4), however, this may have also been due to residual streptomycin sulfate decreasing the sensitivity of the BioRad protein assay, such that the protein content was underestimated.

	DNA (μg/mg prot)		Protein (mg/ml)		
	Before	After	Before	After	% decrease
Control	3.96	2.52	3.59	2.81	21.6
Control	2.75	2.82	3.05	2.59	15.3
DSS	36.22	2.89	2.63	2.19	16.7
DSS	33.70	2.83	2.17	1.82	16.4

Table 5.4: Effects of streptomycin sulfate precipitation on the DNA and protein content of mucosal homogenate supernatants from control and DSS-exposed mice. Results are from two control and two inflamed samples. The protein and DNA content were determined after TCA precipitation of the samples followed by washes with TCA to remove excess streptomycin sulfate, as described in detail in the methods.

After removal of DNA by this method, no difference was observed in the protein carbonyl content of control mucosa and mucosa from mice with DSS-induced colitis (8.43±0.59 vs 7.80±1.05 nmol/mg protein respectively, p=0.16 n=8 & 10). (Figure 5.9). In these samples, a decrease of 13.1% in total reduced thiol content of the supernatants was observed from 133.4±15.0 to 115.9±23.7 nmol/mg protein, although it was not

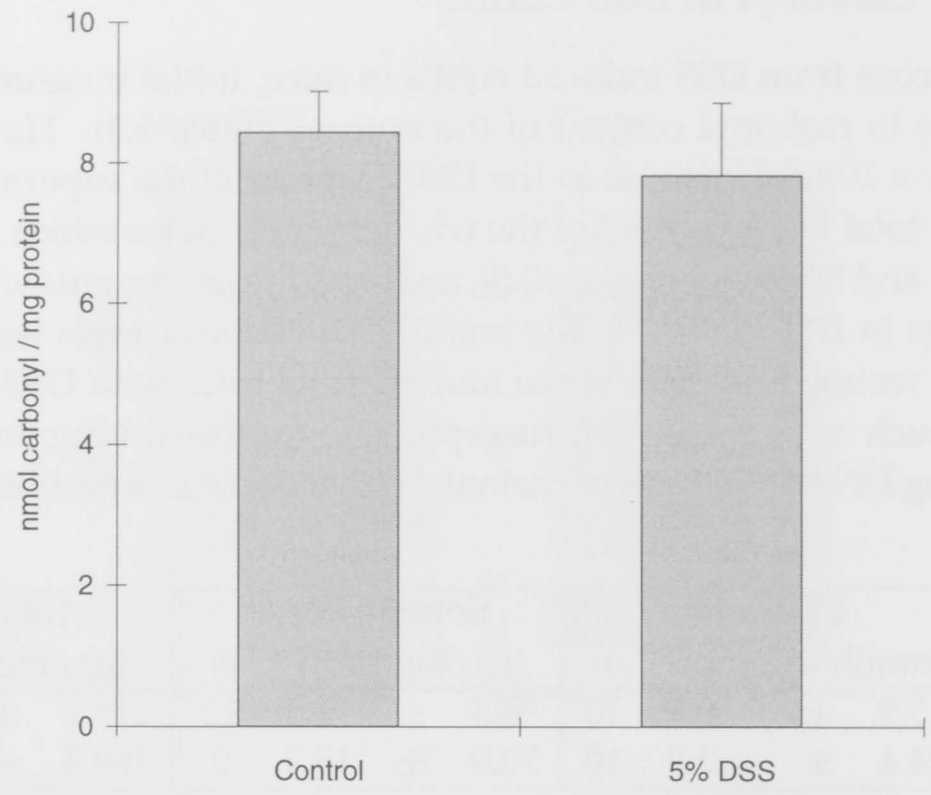


Figure 5.9: Protein carbonyl content in in the colonic mucosa of control mice (n=10) and mice exposed to 5% DSS (n=8). after the removal of soluble DNA by streptomycin sulfate precipitation. P=0.16

significant by Student's t test (n=10 and n=8 respectively, P=0.097). This decrease in total reduced thiols was smaller than that observed in the previously reported experiment, of 25% (Figure 4.5).

While no increase in the total carbonyl content of mucosal proteins was observed, western blotting analysis of mucosa from control and DSS-exposed mice revealed marked differences in anti-DNP signal intensity from several protein bands, indicating increased carbonyl content. In particular, bands of size 48, 79, 95 and 131 kDa were consistently darker in samples from mice exposed to DSS, although on other occasions, the 79, 95 and 131 kDa proteins were not as prominent as in the samples displayed (Figure 5.10). These bands correspond to several of the proteins observed during the *in vitro* studies to be susceptible to carbonyl oxidation. Proteins of size 47 and 131 kDa bands were observed in mucosa exposed to iron/ascorbate (Figure 5.5), and many proteins between 45 and 100 kDa were observed to be oxidised by HOCl (Figure 5.6).

In an attempt to identify these susceptible proteins, the 48 kDa band was selected for protein sequence analysis because of its prominence and relative abundance. However, this revealed that this band comprised at least three proteins, thus confounding identification of the oxidised protein(s).

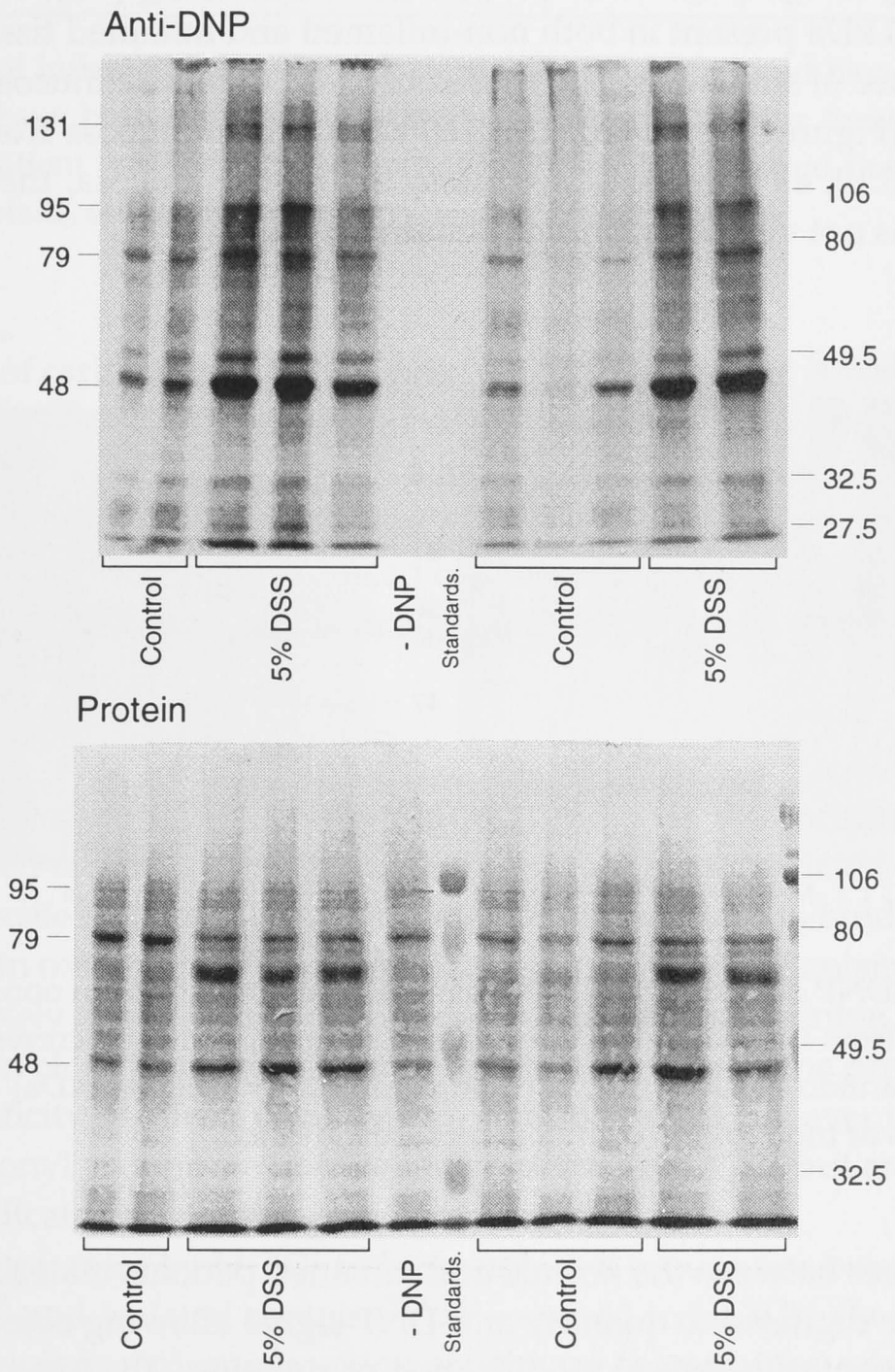


Figure 5.10: Colonic mucosal proteins from control mice and mice exposed to 5% DSS: detection of carbonyl groups by immunoblotting with anti-DNP antibody (top) and corresponding staining for protein with amido black (bottom). Molecular weights (kDa) of some carbonyl modified proteins (left) or the positions of molecular weight markers (right) are indicated. -DNP lane contains underivatised proteins for negative control.

5.3.4 Protein Carbonyl in IBD

Western blotting analysis of biopsy material from IBD patients was performed to determine if carbonyl formation might be implicated in IBD. The amount of material in biopsies is not sufficient for spectrophotometric determination of carbonyls. The western blotting analysis revealed several DNP-containing proteins of size 49, 66, 120, 131 and 140 kDa present in both non-inflamed and inflamed tissue (Figure 5.11). These proteins are of similar sizes to those observed in mouse mucosa exodised with iron/ascorbate (Figure 5.5, 49, 120, 131, 140 kDa) and in mucosa from mice exposed to DSS (Figure 5.10, 48 and 131 kDa). As in the mouse mucosa, the high molecular weight band was not visible by protein staining.

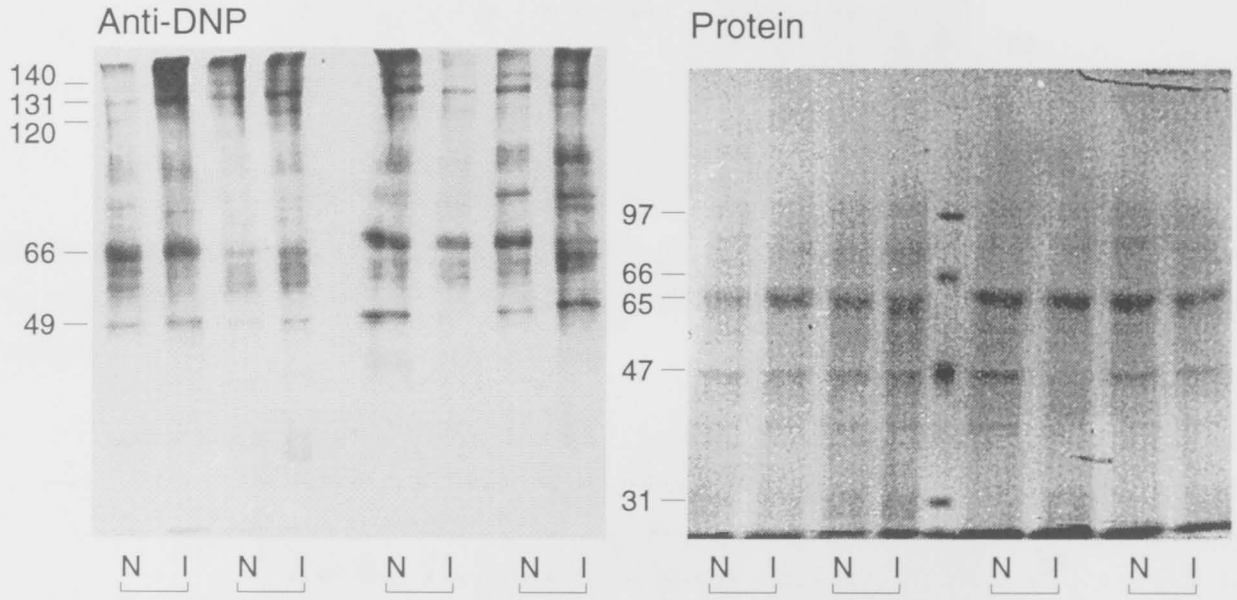


Figure 5.11: Anti-DNP detection (left) of human mucosal proteins from non-inflamed (N) and inflamed (I) sites from IBD patients. Paired samples are adjacent, and corresponding staining for protein with amido black is shown (right). Molecular weights (kDa) of some carbonyl modified proteins or molecular weight markers are indicated.

The differences between the normal and inflamed patients material were variable, so the blots were digitised to quantify anti-DNP signal allowing objective assessment of the overall extent of carbonyl modification of proteins. The outcome of digitising and determining the ratio of the signal from inflamed to non-inflamed pairs is shown in Table 5.5. In 9 pairs of biopsies from IBD patients the ratio was greater than 1.05 in 4 pairs, unchanged in 1 pair, and less than 0.95 in 4 pairs. Separation of results according to disease or the treatments being taken by the patients did not reveal any pattern of protein oxidation associated with disease type or therapy.

5.4 Discussion

RONS have been implicated in the pathogenesis of IBD, however, there are very few reports of protein oxidation in IBD or experimental colitis. Increased protein oxida-

Ratio Category	Total			Disease		Treatment	
	n	mean	range	UC	CD	Nil	+
>1	4	2.04	1.14–3.12	3	1	2	2
=1	1	1.01	–	1	0	0	1
<1	4	0.63	0.57–0.77	3	1	2	2

Table 5.5: Ratio of inflamed:non-inflamed anti-DNP signal of paired biopsies from IBD patients. Samples have been categorised according to disease type or therapy at the time of biopsy. +: The patient was receiving aminosalicylate or corticosteroid therapy or both. For further patient details, see methods section.

tion in the form of carbonyl groups has been reported in several diseases where RONS are implicated.[274–278] A recent paper has described a western blotting method for the detection of protein carbonyls[279] which may reveal specific proteins that are susceptible to oxidation. Oxidation of colonic mucosal proteins in the form of carbonyl groups was investigated *in vitro*, in DSS-induced colitis and in IBD, using spectrophotometry and the novel western blotting approach to identify particular targets of oxidation.

5.4.1 *In vitro* Carbonyl Formation

Oxidation of mucosa by iron/ascorbate, HOCl, and, at high concentrations, $^{\bullet}NO$ can result in increased levels of protein carbonyl groups. Iron-mediated carbonyl formation is a site-specific reaction, where iron may bind to metal-ion binding sites on proteins and result in oxidation of nearby residues.[281] By this mechanism, the amino acid residues most likely to form carbonyl derivatives are lysine, arginine, proline and histidine residues in close proximity to the metal binding site of the protein.[282] Because of this site specificity, reduced thiols can be spared even in the event of carbonyl formation.[269] Carbonyl groups are not the only outcome of MCO, but are considered the most reliable indicator of MCO of proteins *in vitro*.[105]

Oxidation of proteins by HOCl to form carbonyl groups has been reported previously for cells[236] and isolated proteins[229,283] exposed to HOCl. Carbonyl formation by HOCl can result from decomposition of primary chloramines, which may form on lysine residues or on terminal amino groups:[270,284]

$$\begin{aligned} RCH_2NH_2 + HOCl &\longrightarrow OH^- + RCH_2NHCl \\ RCH_2NHCl &\longrightarrow HCl + RCH{=}NH \\ RCH{=}NH + H_2O &\longrightarrow RCH{=}O + NH_3 \end{aligned}$$

Thiols are known to be very susceptible to oxidative attack by HOCl,[88,270] thus it is no surprise that the formation of carbonyls on mucosal proteins was preceded by depletion of reduced thiols. Thiol depletion before carbonyl formation has been observed by others,[236,271] and further, administration of thiols has reduced carbonyl formation in some circumstances.[271,275]

H_2O_2 and $^{\cdot}NO$ have both been reported to generate protein carbonyls in different circumstances. H_2O_2 can participate in site-specific, metal-catalysed oxidation by reacting with iron (Equation 1.3) and allowing $^{\cdot}OH$ generation to occur.

$$Fe^{2+} + H_2O_2 \longrightarrow {}^{\cdot}OH + OH^- + Fe^{3+}$$

Many investigators use this model for *in vitro* studies of MCO.[281] While in the mucosal homogenates there should have been sufficient iron available for such reactions to occur, catalase in the tissue may have consumed the H_2O_2 thus preventing any form of oxidation. This is supported by the surprisingly small decrease in total reduced thiols, and by the amount of pressure built up in the tubes during the incubation period, probably due to liberation of O_2. $ONOO^-$ has also been reported to generate carbonyl groups on bovine serum albumin (BSA) and in rat lung homogenates.[104] While the oxidant used here was $^{\cdot}NO$ rather than $ONOO^-$, formation of $ONOO^-$ or other reactive nitrogen species is possible at the highest concentration used (Section 6.4.2.1) which may account for the carbonyl formation. As was observed in the present study with $^{\cdot}NO$, carbonyl formation on BSA by $ONOO^-$ was also preceded by depletion of reduced thiols.[104]

The use of western blotting analysis for protein carbonyls provides further information about the protein oxidation occurring. Oxidation of mucosal proteins with iron/ascorbate resulted in carbonyl formation on many proteins without crosslinking or degradation. This is consistent with the changes in plasma observed by Shacter *et al.*[279] The inactivation of enzymes and subsequent degradation by proteases after MCO has been studied extensively, and aggregation or fragmentation has not been reported.[281]

The lack of fragmentation with iron/ascorbate contrasted the pattern of oxidation induced by HOCl. Carbonyl formation was first observed on selected proteins, followed by extensive crosslinking of proteins at higher HOCl concentrations. Crosslinking of proteins by HOCl is a well recognised phenomenom, with studies reported on immune complexes, fibronectin and LDL, and is suggested to play a role in inflammatory processes such as arthritis and athersclerosis.[229,283,285] This crosslinking may occur *via* several mechanisms including dityrosine formation[228] and decomposition of chloramines on lysine residues.[283] High concentrations of HOCl also caused fragmentation which may be a result of direct oxidant cleavage or protease activity. Protein staining indicated only a small amount of material was degraded, but it was very rich in carbonyl groups, resulting in intense smearing of the anti-DNP signal.

5.4.2 *In vivo* Carbonyl Formation

The possibility of iron-mediated injury being involved in colitis has been described previously (Section 1.3.1). While in normal tissues, iron-mediated tissue damage is considered very unlikely because of the antioxidant defences of SOD, catalase and metal-ion binding proteins, in a diseased tissue such as the inflamed and ulcerated intestinal mucosa, exposure to luminal iron, mucosal bleeding and the presence of

stimulated inflammatory cells may significantly increase the availability of iron and oxidants to mediate MCO.[137] At the same time, the production of oxidants, such as HOCl and $ONOO^-$, by inflammatory cells may also result in oxidation of proteins. *In vitro*, exposure of mucosal proteins to these oxidising systems was shown to lead to carbonyl formation. These techniques were then applied to mucosa from the DSS mouse model of colitis and IBD patients.

Analysis of the mucosa from control and DSS-exposed mice was hampered by interference from the extraordinary differences in the soluble DNA content of the homogenate supernatants between control and inflamed tissue (Table 5.3). The increase in supernatant DNA may be due to necrosis and tissue degradation within inflamed sites, or increased fragility of the nuclear and chromosome structures which are then disrupted during homogenising of the tissue. A more gentle homogenising or tissue mincing as suggested by Reznick and Packer[286] may have reduced this problem.

Spectrophotometrically, after removal of DNA, the protein carbonyl content of the mucosa was unchanged between inflamed and control tissue (Figure 5.9). Investigation of protein carbonyls in other diseases where oxidants may be implicated report increases ranging from 50–200%.[274–278] While increases in total carbonyls of this magnitude were not observed in DSS-induced colitis, western blotting with anti-DNP detection of proteins revealed differences in the carbonyl content of specific proteins. In particular, proteins of molecular weights 48, 79, 95 and 131 kDa gave more intense anti-DNP signals in the inflamed mucosa compared to control tissue. The occurrence of increased anti-DNP signal on proteins of similar sizes in the mucosa exposed to iron/ascorbate or HOCl *in vitro*, further suggests that these differences are representative of protein oxidation within the inflamed tissue.

Identification of the 48 kDa modified protein was attempted, however, the identity remains elusive as this band contains at least three comigrating proteins. Further separation of the band constituents, either by gradient gel or two-dimensional electrophoresis, may enable the oxidised protein to be identified. Work is in progress to isolate sufficient material of the other oxidised proteins for sequence analysis. We would speculate that these proteins have metal binding sites or contain exposed lysine and arginine residues, which may explain their susceptibility to carbonyl oxidation.

Preliminary studies of mucosal biopsies from IBD patients by western blotting detected several proteins in both inflamed and control biopsies, however there were not consistent differences between the inflamed and non-inflamed mucosa pairs for overall carbonyl content or for specific protein bands. The increased anti-DNP signal from 4 out of 9 pairs compared to a decrease in 4 out of 9 is not suggestive of significant carbonyl oxidation of proteins occurring in the inflamed lesion. While amino-salicylates used in IBD treatment have many antioxidant properties *in vitro* which may contribute to their mechanism of action (Section 1.3.4), there was also no pattern of protein oxidation as carbonyl groups in patients receiving aminosalicylates compared to untreated patients. The pattern of proteins observed by anti-DNP detection of human colonic mucosa was similar to that observed in the mouse mucosa, suggesting that further investigations in the mouse may be relevant to the human disease. Identification of the modified proteins from the mouse studies may allow more specific functional assays

for potential targets to be performed on mouse and human tissue, thus indicating if loss of protein activity which may be due to carbonyl oxidative modification occurs in the inflamed mucosa.

In vitro, carbonyl formation by HOCl and $^{\bullet}NO$ was preceded by depletion of thiols. The small change in reduced thiols observed in the mucosa from DSS-induced colitis indicates that, on average, extensive carbonyl formation from HOCl or $^{\bullet}NO$ oxidation would not be expected. However, induction of colitis using DSS in CBA/H mice results in a disease with intense inflammatory foci and ulcerations, surrounded by comparatively normal tissue (Figure 2.3). Within the focal lesions, thiol and antioxidant depletion may be sufficient to enable carbonyl formation to occur, but generating insufficient to be detected by the total carbonyl method. In contrast, carbonyl formation *via* a MCO mechanism may not require thiol depletion, however iron, from mucosal bleeding and exposure to faecal contents where the mucosal barrier has been compromised, and the oxidants required to mediate oxidation would also be most abundant within the inflammatory foci.[137] These phenomena are not an issue for the biopsies from IBD patients, as they have been selected to represent acutely inflamed and non-inflamed areas of mucosa. Even in tissue thus selected, consistent differences in carbonyl / anti-DNP detection were not observed (Figure 5.11).

Oxidation of proteins, including carbonyl formation, is considered to mark proteins for degradation by proteases selective for oxidised proteins.[106,287] While not studied extensively *in vivo*, it has been demonstrated that these selective proteases can be induced by oxidative stress.[274] Therefore, while oxidation of proteins with carbonyl generation may occur, especially around the inflammatory foci where HOCl concentrations and iron availibility would be elevated, the oxidised proteins may be degraded rapidly by proteases induced by oxidative stress and released by activated phagocytes. Such an increase in protein turnover would make it difficult to determine the amount and significance of protein oxidation occurring *in vivo*.

5.4.3 Conclusion

Protein carbonyls can be generated on mucosal proteins by oxidation using iron-mediated oxidation, HOCl or $^{\bullet}NO$. The outcome of oxidation by these processes is different; modifications associated with oxidation such as crosslinking and fragmentation were highlighted by the western blotting analysis of oxidised mucosa. While total protein carbonyls do not appear to be significantly increased in DSS-induced colitis, western blotting analysis indicated several proteins which may be particular targets for carbonyl oxidation. Western blotting of IBD biopsies showed several candidate proteins of similar molecular weight to those observed in the inflamed mouse mucosa. Identification and further analysis of the mucosal proteins susceptible to carbonyl-modification may lead to a better understanding of the contribution of oxidants to the tissue injury in the colonic mucosa in IBD.

Chapter 6

The Role of Nitric Oxide

6.1 Introduction

Considerable indirect evidence has been reported suggesting a role for $^{\bullet}NO$ in the pathogenesis of IBD. Increased nitrite levels in rectal dialysates from patients with active ulcerative colitis have been observed.[288] More recently, elevated levels of citrulline[133] and NOS activity[134] in biopsies of inflamed mucosa, and 100-fold higher luminal $^{\bullet}NO$ gas concentration in ulcerative colitis patients[132] have been found. This has prompted investigations into the involvement of $^{\bullet}NO$ production in the pathogenesis and tissue injury of IBD.

In inflammatory lesions, the likely source of increased production of $^{\bullet}NO$ is increased expression of inducible NOS (iNOS) by activated macrophages and neutrophils. The proinflammatory cytokines interferon γ and tumour necrosis factor α, and bacterial products such as LPS and fMLP, are capable of inducing iNOS expression in macrophages and neutrophils, a process which plays an important role in the cytotoxic capabilities of macrophages.[66,289] Under these circumstances, $O_2^{\bullet-}$ production by the membrane NADPH oxidase is also stimulated, and thus $ONOO^-$ may be generated by the reaction of $O_2^{\bullet-}$ and $^{\bullet}NO$ (Equation 1.15):[94,95]

$$^{\bullet}NO + O_2^{\bullet-} \longrightarrow ONOO^-$$

$^{\bullet}NO$ can participate in a host of reactions with biomolecules to mediate physiological and pathophysiological changes. Neurotransmission and vasodilation are among the physiological processes with which $^{\bullet}NO$ is involved, while oxidation of thiols and inactivation of ribonucleotide reductase are examples of its ability to interfere in normal function of cells.[66,71] $ONOO^-$ can decompose to generate $^{\bullet}OH$ (Equation 1.16) and can itself oxidise thiols irreversibly.[96,290] While many of these outcomes are not specific for $^{\bullet}NO$ or $ONOO^-$, $ONOO^-$ is also capable of nitrating aromatics including tyrosine (Figure 6.1). The formation of nitrotyrosine is considered specific evidence of $ONOO^-$-mediated oxidation.[291,292] A recent immunohistochemical study has revealed elevated levels of nitrotyrosine in TNBS-induced ileitis in guinea pigs, where the NOS inhibitors, nitro-L-arginine methyl ester (L-NAME) and aminoguanidine, were effective in alleviating colitis.[172]

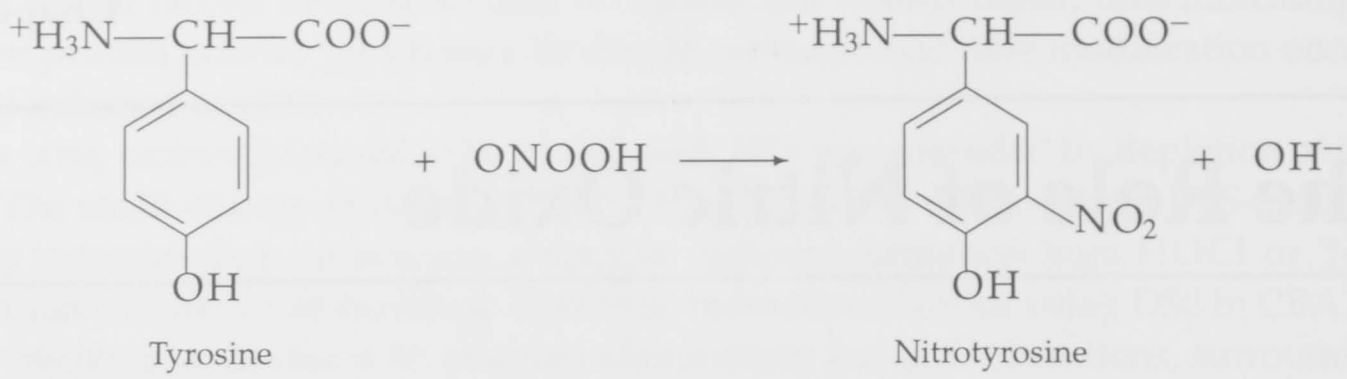


Figure 6.1: Reaction of tyrosine with ONOOH and formation of nitrotyrosine.[292]

NOS inhibitors can provide further information about the role of ·NO in disease. Currently used NOS inhibitors are substrate analogues which have varying specificities for the different NOS isoforms (Section 1.2.1.2) and can be administered so as to cause the systemic inhibition of NOS. L-NAME inhibits both iNOS and cNOS (constitutive NOS) and is commonly used in animal studies. Using this strategy, a pathogenic role for ·NO has been suggested in inflammatory disorders such as model arthritis,[293,294] and also in several models of intestinal inflammation, including TNBS-induced ileitis in guinea pigs and chronic granulomatous colitis in rats.[135,171]

The aggressive inflammation with neutrophil and macrophage infiltration in this model suggests that excessive ·NO production by these cells may contribute to the tissue injury in acute DSS-induced colitis. To investigate this possibility, two experimental approaches were take: the effect of the NOS inhibitor L-NAME on the disease course was investigated; and the nitrotyrosine content of mucosal proteins was examined by western blotting.

6.2 Methods

6.2.1 Induction of Colitis and Inhibition of NOS Activity

For the investigation of NOS inhibition, colitis was induced in male, 9–10 week old balb/c mice by administration of 5% DSS in the drinking water for 6 days. The recovery of the mice was observed for a further 10 days, during which time they drank water. Five groups of 8–10 mice were involved in this experiment of which three groups received the NOS inhibitor L-NAME. In two groups, L-NAME was adminin-stered in the drinking water at 100 μg/ml either without DSS or with DSS, which delivered 10.7–12.7 mg L-NAME/kg/day. The third group recevied L-NAME at an equivalent dose of 12 mg/kg/day in two i.p. injections. L-NAME treatment commenced with DSS administration and continued until the end of the experiment. This dose of L-NAME has been shown by others to lead to systemic inhibition of NOS.[135] The remaining two groups received either DSS alone or no treatment. Diarrhoea, rectal bleeding, bodyweight and fluid consumption were monitored daily as described in Chapter 2.

For studies on nitrotyrosine formation, colitis was induced in CBA/H mice with 5% DSS, and colonic mucosal homogenates from control and DSS-exposed mice was collected as described previously (Section 2.2.2).

6.2.2 Exposure of Mucosal Proteins to Nitric Oxide

Mouse colonic mucosal homogenates were exposed to $^{\bullet}NO$ *in vitro* as described previously (Section 5.2.1). In brief, mucosa collected from male CBA/H mice was homogenised, diluted to 5 mg tissue/ml, and exposed to 0, 0.1, 1 and 10 mM diethylamine NONOate (Cayman Chemical Company) at 37°C for 30 min. NONOate, prepared in sterile PBS pH 8.5, decomposes to release $^{\bullet}NO$ at neutral pH, with a half life of 2.1 min at pH 7.4. Thus 30 min was sufficient for all $^{\bullet}NO$ to be released.[207] 50 μl of whole homogenate was aliquotted and frozen on dry ice for examination of nitrotyrosine-containing proteins by western blotting.

6.2.3 Collection of Human Biopsies

Paired mucosal tissue specimens were obtained by biopsy of histologically normal (non-inflamed) and inflamed sites in patients with inflammatory bowel disease as described in Section 5.2.3. Aliquots of biopsy homogenate were used for nitrotyrosine analysis.

6.2.4 Western Blotting For Nitrotyrosine

Mucosal homogenates from mice exposed to DSS, human biopsies, and mouse mucosal homogenates exposed to $^{\bullet}NO$ *in vitro* were examined for proteins containing nitrotyrosine by western blotting. The protein content of homogenates was determined by the BioRad protein assay (Section 2.2.3). Aliquots of homogenates were mixed with reducing sample buffer (0.125 M Tris-HCl pH 6.8, 20% glycerol, 4% SDS, 1 M β-mercaptoethanol, 0.1% bromophenol blue) and 10 μg of mucosal proteins were subjected to 9% SDS-PAGE (BioRad Mini-Protean II gel system). Positive controls of nitrotyrosine-containing molecular weight markers (Upstate Biotechnology Incorporated, New York) and mucosal proteins exposed to $^{\bullet}NO$ *in vitro* were also loaded on the gels. This was followed by electroblotting (BioRad Mini Trans-blott transfer cell) onto nitrocellulose (Hybond, Amersham).

For mouse mucosa samples, membranes were blocked with skim milk and incubated with polyclonal rabbit anti-nitrotyrosine antibody (Upstate Biotechnology Incorporated, New York) 0.5 μg/ml, followed by a horse radish peroxidase-conjugated sheep anti-rabbit antibody (Silenus, diluted 1/10,000). Proteins containing nitrotyrosine were visualised by enhanced chemiluminescence (Amersham ECL kit) and exposure of film (Hyperfilm-ECL, Amersham). When detecting the human samples, the above procedure was followed using 2 μg/ml anti-nitrotyrosine antibody, 1/3,000 dilution of sheep anti-rabbit antibody, and a 5-fold longer exposure to ECL reagents.

6.3 Results

6.3.1 Effect of L-NAME on Disease Course

Mice treated with the NOS inhibitor L-NAME showed slight differences in the course of disease compared to control DSS mice (Figure 6.2). L-NAME treatment, by either oral or i.p. delivery, slowed the development of diarrhoea by 1–2 days, peaking on days 7–8 compared to day 6 in untreated mice, followed by faster recovery from diarrhoea. The differences in rectal bleeding are more complex, with oral L-NAME increasing the number of mice with the symptom on days 6–7 compared to controls, while mice receiving L-NAME i.p. developed a late recurrence of bleeding on days 14–15.

Treatment of mice with L-NAME altered the fluid consumption of both control mice and mice receiving dextran (Figure 6.3). Control mice receiving oral L-NAME consumed 20% less fluid compared to untreated control mice. Inclusion of 5% DSS in the water decreased fluid intake by 23%, and treatment with L-NAME by oral or i.p. delivery resulted in reductions in drinking rates of 30% and 47% respectively. The latter figure may in part have been replaced by the injected fluid, which was 200 μl per day per mouse, or 0.05 ml/g mouse (5.9% of the intake of control mice) cumulative over the 6 day period of DSS administration. Replacement of DSS with water resulted in all DSS-treated groups consuming approximately double the volume of fluid on day 6–7, resulting in the increase in body weights recorded on day 7.

Mortalities occurred only in the mice exposed to DSS receiving oral L-NAME (Figure 6.4). In this group, 4 out of 8 mice died from severe disease between days 8 and 12. This affected the average symptom score in this group as the mice with most severe symptoms were progressively removed. Average body weights were also influenced by the deaths, with an unusual increase in average body weights for this group being observed on day 12.

6.3.2 Western Blotting Analysis of Nitrotyrosine

Exposure of mouse mucosal homogenates to 10 mM $^{\cdot}NO$ *in vitro* resulted in nitrotyrosine formation on many proteins. *in vitro* (Figure 6.5 A). At lower concentrations of $^{\cdot}NO$, however, the nitrotyrosine signal did not differ from untreated mucosal proteins, even when a much longer exposure of the film was used (Figure 6.5 B). Analysis of reduced thiols in these homogenates showed that $^{\cdot}NO$ decreased the total thiol content of the mucosal homogenate in a dose related manner (Figure 6.6). At 10 mM $^{\cdot}NO$, reduced thiols were depleted by 90%.

Similarly, western blotting analysis of mucosal homogenates from control mice and mice exposed to 5% DSS had a very weak signal compared to the 10 mM $^{\cdot}NO$-treated homogenates. Long exposures revealed many bands in both control and DSS samples, with no marked difference between the two groups (Figure 6.7).

Western blotting of human biopsy samples showed similar results. Four pairs of biopsies, non-inflamed and inflamed, from IBD patients with active disease were examined (two untreated UC patients, two CD patients receiving steroids, age range

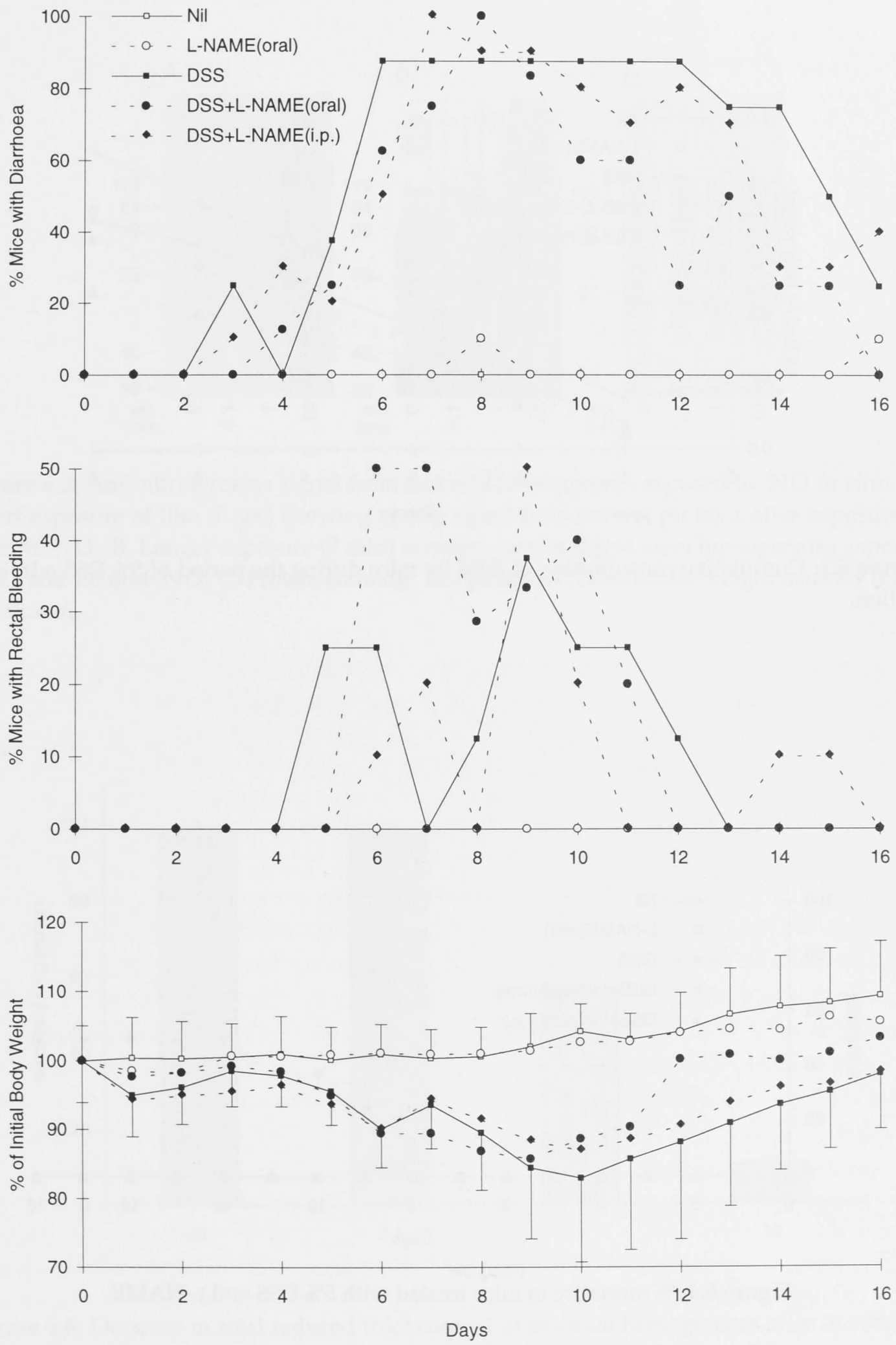


Figure 6.2: Disease course of mice exposed to 5% DSS for 6 days, followed by water, and treated with L-NAME, 12 mg/kg/day either orally or i.p. Figure shows the percentage of mice observed with diarrhoea (top) and rectal bleeding (centre), and body weight has been expressed as % of initial weight (bottom). n=8–10 mice/group.

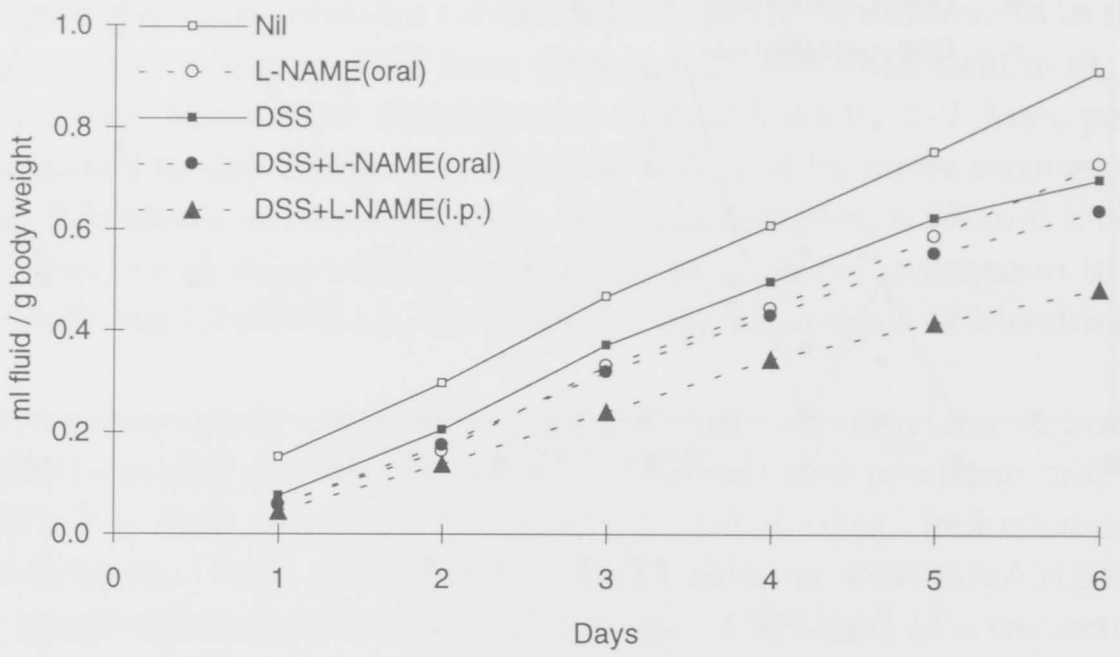


Figure 6.3: Cumulative consumption of fluid by mice during the period of 5% DSS administration.

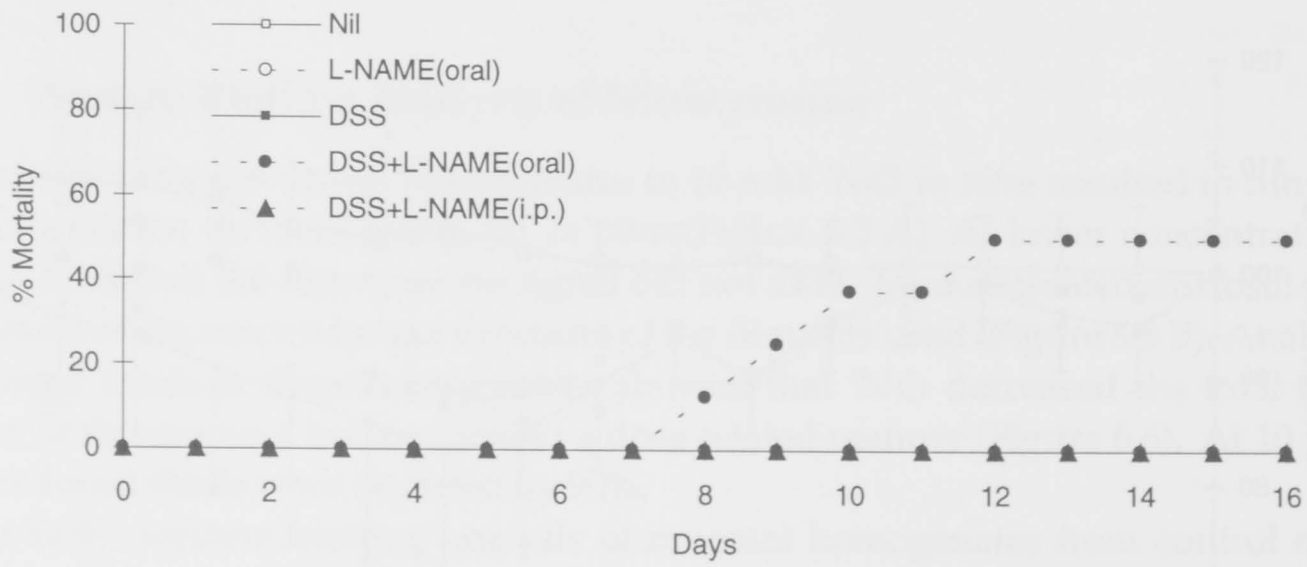


Figure 6.4: % mortality in mice treated with 5% DSS and L-NAME.

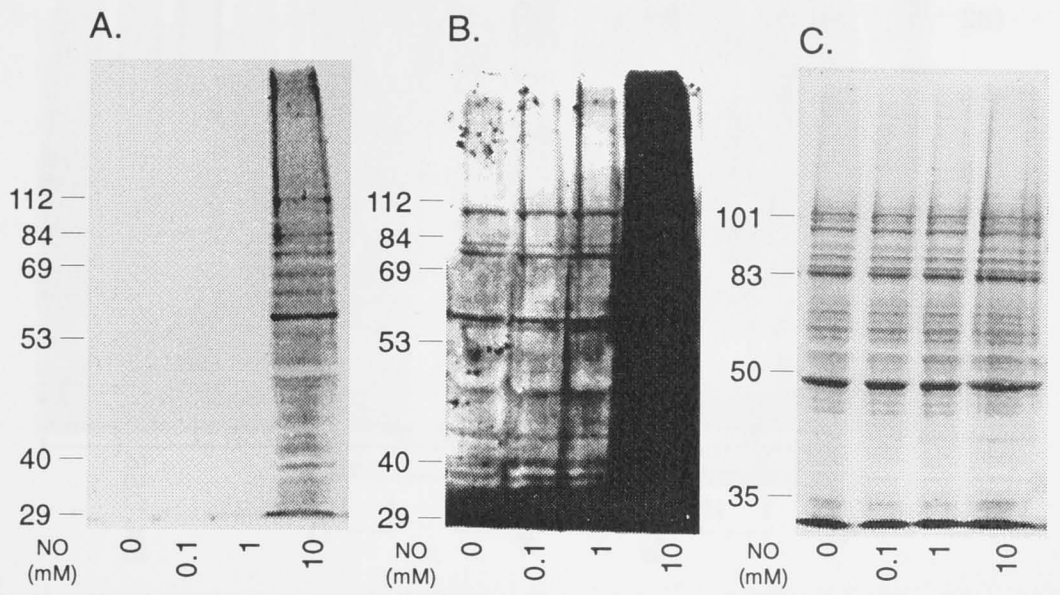


Figure 6.5: Anti-nitrotyrosine signal from mucosal homogenates exposed to ˙NO *in vitro*. A. Short exposure of film (5 sec) showing strong signal from several proteins after exposure to 10 mM ˙NO. B. Longer exposure (7 min) showing lack of signal from homogenates exposed to 0.1 and 1.0 mM ˙NO. C. Protein staining. The positions of molecular weight markers (kDa) are shown.

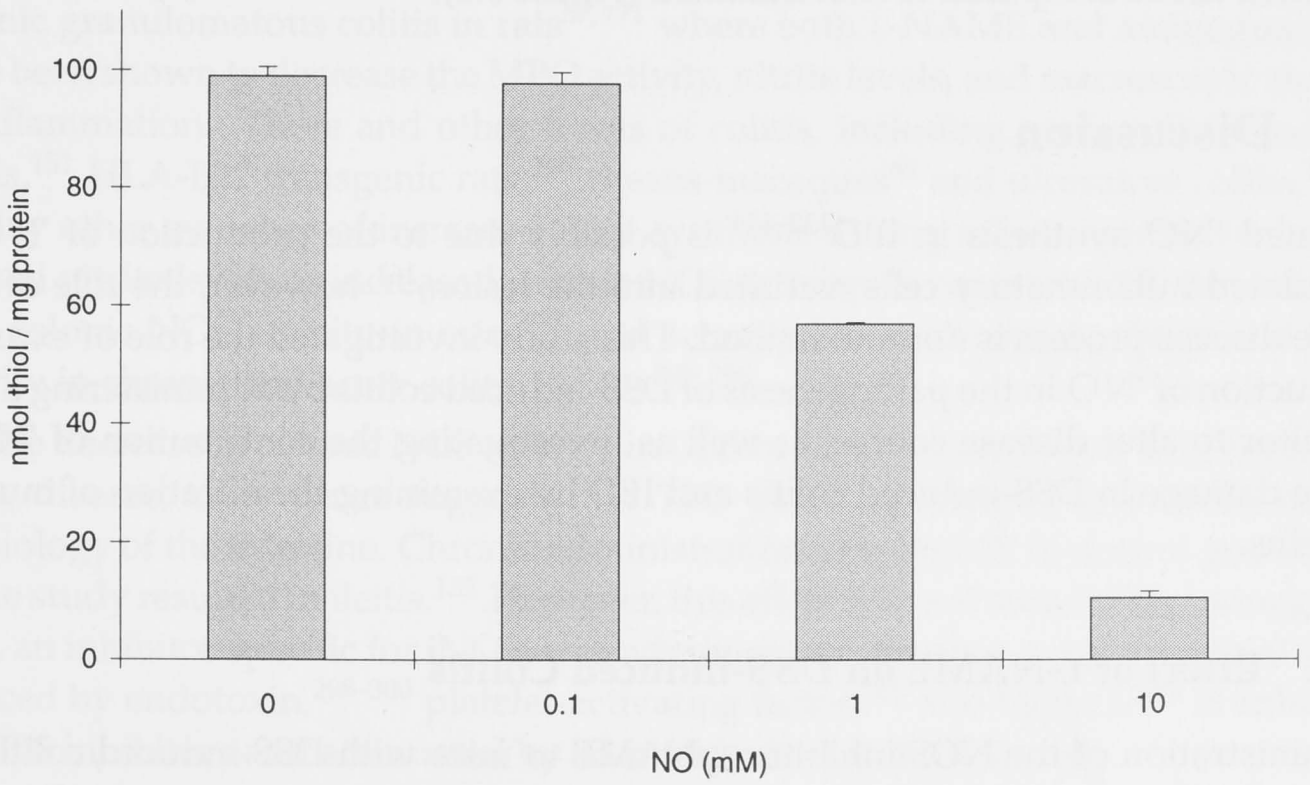


Figure 6.6: Decrease in total reduced thiol content of mucosal homogenates after *in vitro* exposure to increasing concentrations of ˙NO. (Data reproduced from Figure 5.3.)

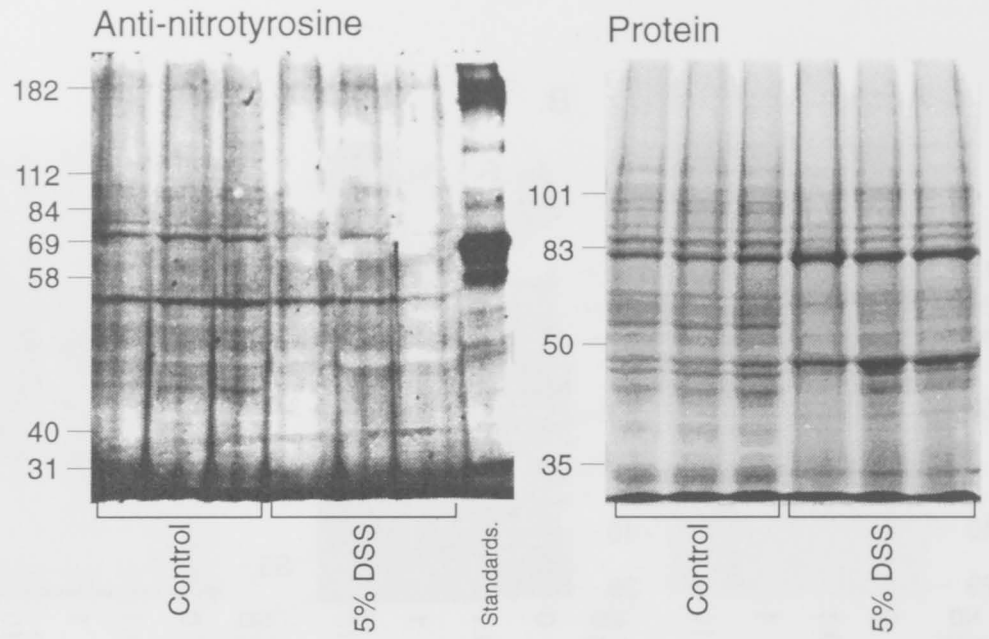


Figure 6.7: Detection of nitrotyrosine on colonic mucosal proteins from control mice and mice with DSS-induced colitis. Anti-nitrotyrosine signal (left) including nitrotyrosine-containing standards, and protein staining (right). The positions of molecular weight markers (kDa) (standard and nitrotyrosine-containing) are shown.

23–45 years). Using higher antibody concentrations and more ECL reagent in the detection process (resulting in higher background signal also), many bands were detected in both non-inflamed and inflamed tissue, but with no greater intensity in the inflamed tissue compared to non-inflamed (Figure 6.8).

6.4 Discussion

Elevated $^{\bullet}NO$ synthesis in IBD[132–134] is possibly due to the production of $^{\bullet}NO$ by stimulated inflammatory cells recruited into the lesion,[161] however, the role of $^{\bullet}NO$ in the disease process is not understood. This study investigated the role of excessive production of $^{\bullet}NO$ in the pathogenesis of DSS-induced colitis by administering a NOS inhibitor to alter disease course, as well as investigating the contribution of $^{\bullet}NO$ to tissue damage in DSS-induced colitis and IBD by examining the nitration of mucosal proteins.

6.4.1 Effect of L-NAME on DSS-induced Colitis

Administration of the NOS inhibitor L-NAME to mice with DSS-induced colitis did not markedly reduce the severity of disease. While a slight improvement in diarrhoea was apparent in L-NAME treated animals, this may be due to the 10–20% decrease in consumption of DSS, as fluid intake was supressed by L-NAME. While the numbers of animals in these groups were small, the findings are in agreement with other recent reports of a lack of benefit from NOS inhibition in DSS-induced colitis. NOS inhibition

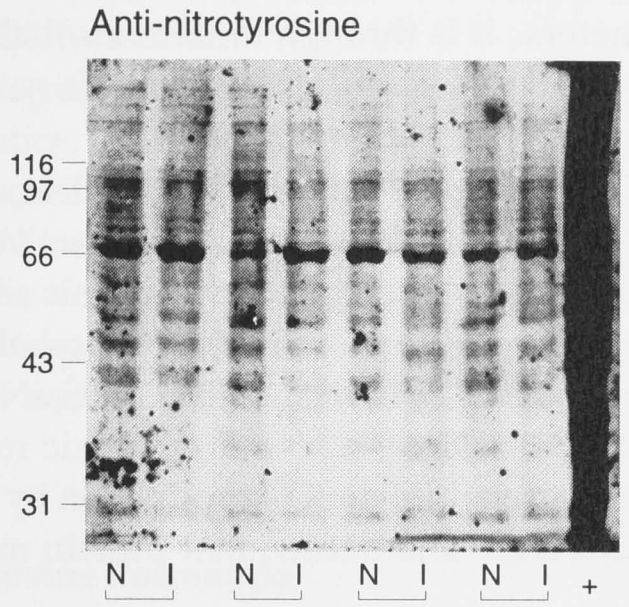


Figure 6.8: Anti-nitrotyrosine signal ~~(left) and protein staining (right)~~ from paired IBD mucosal biopsy homogenates. N - non-inflamed, I - inflamed biopsies from the same patient, + - positive control of mouse homogenates exposed to 10 mM $^{\bullet}$NO *in vitro*. The position of molecular weight markers (kDa) are shown.

by either aminoguanidine or L-NAME did not attenuate the chronic colitis induced by oral administration of DSS to mice or rats[295] and L-NAME induced a marked increase in the severity of mucosal lesions in acute DSS colitis in rats.[296] This is in contrast to the TNBS model of chronic ileitis in guinea pigs,[135] TNBS-induced colitis[136] and chronic granulomatous colitis in rats[57,171] where both L-NAME and aminoguanidine have been shown to decrease the MPO activity, nitrite levels, and macroscopic changes of inflammation. These and other forms of colitis, including peroxynitrite-induced colitis,[151] HLA-B27 transgenic rats,[297] rhesus-macaques[48] and ulcerative colitis,[134,288] display other evidence of increased $^{\bullet}$NO synthesis during inflammation, such as increased nitrite levels or increased colonic NOS activity. In contrast, DSS alone did not alter colonic NOS activity in mice[295] and actually lowered colonic cNOS and iNOS activity in chronic and acute colitis in rats.[295,296]

The decrease in colonic NOS activity in DSS-induced colitis in rats may contribute to the disease, as evidence suggests that $^{\bullet}$NO plays a protective role in the normal physiology of the intestine. Chronic administration of L-NAME to control guinea pigs in one study resulted in ileitis.[135] However, this effect was not seen when aminoguanidine, an inhibitor specific for iNOS, was administered. Further, acute intestinal injury induced by endotoxin,[298–300] platelet-activating factor,[301] and hypoxia[302] is enhanced by NOS inhibitors and attenuated by administration of $^{\bullet}$NO donors .

This wide range of responses to manipulations of $^{\bullet}$NO production in different circumstances is currently rationalised by discussion of the source of the $^{\bullet}$NO and the consequent function it was proposed to have. Under physiological circumstances, infusion of L-NAME increases leukocyte adhesion and migration,[303] microvascular permeability[304] and intestinal epithelial permeability,[305] indicating that $^{\bullet}$NO produced

constitutively by cNOS in the endothelial or epithelial cells has an important role in maintaining these parameters. It is through these roles that $^{\cdot}NO$ is thought to have its protective effects in situations of acute intestinal injury, such as that induced by endotoxin, platelet-activating factor and hypoxia.

While increased $^{\cdot}NO$ production may be beneficial in acute disease, in chronic conditions, when both iNOS and cNOS are likely to be active, elevated production of $^{\cdot}NO$ has the potential to result in tissue injury. In chronic situations, increased iNOS expression may result from exposure of infiltrating macrophages and neutrophils to proinflammatory cytokines and LPS. In contrast to cNOS, iNOS produces large quantities of $^{\cdot}NO$, as required for its cytostatic and cytotoxic role in nonspecific immunity.[71,289] These conditions also stimulate $O_2^{\cdot-}$ production by inflammatory cells, with potential generation of $ONOO^-$ and subsequent protein modification and mucosal damage.

The role of $^{\cdot}NO$ in acute or chronic DSS-induced colitis, as suggested so far by NOS-inhibitor studies, remains unclear. Investigations of the effect of $^{\cdot}NO$ donors, such as S-nitroso-N-acetyl-penicillamine, may help elucidate a potentially protective role for $^{\cdot}NO$ in this disease.*

6.4.2 Nitrotyrosine on Mucosal Proteins

While it was not possible to implicate $^{\cdot}NO$ in acute DSS-induced coitis by the use of the NOS inhibitors, the formation of nitrotyrosine-modified proteins was investigated as an indicator of the involvement of $^{\cdot}NO$, *via* $ONOO^-$, in protein oxidation and tissue damage. Nitrotyrosine is considered a specific indicator of $ONOO^-$-mediated tissue injury[306] and has been associated with protein oxidation in several diseases including ileitis,[172] atherosclerosis,[307] ischaemic rat lung injury[308] and acute lung injury.[309,310] This study investigated nitrotyrosine formation on mucosal proteins after *in vitro* exposure to $^{\cdot}NO$, in mucosa from control mice and mice with DSS-induced colitis, and in paired IBD biopsies.

6.4.2.1 *In vitro* Nitrotyrosine Formation

$^{\cdot}NO$ exposure caused the formation of nitrotyrosine on many mucosal proteins as detected by western blotting. While $^{\cdot}NO$ itself is not capable of nitrating phenolics,[291] the formation of secondary reactive nitrogen species could explain the nitration observed. *In vivo*, nitrotyrosine is considered specific for $ONOO^-$ attack. This requires coincident $^{\cdot}NO$ and $O_2^{\cdot-}$ generation, the later of which may not be generated in sufficient quantities in the system employed in these experiments. Other species capable of nitrating phenolics include $^{\cdot}NO_2$, which is formed from the reaction of $^{\cdot}NO$ with molecular oxygen:

$$^{\cdot}NO + O_2 \longrightarrow 2\,^{\cdot}NO_2 \qquad (6.1)$$

While this pathway of nitration is considered too slow and inefficient to be responsible for nitration of tyrosine *in vivo*, in this situation *in vitro* where the $^{\cdot}NO$ concentration

*These results need to be substantiated with direct measurement of nitrate/nitrite levels.

was very high, and O_2 probably more abundant than $O_2^{\cdot-}$, perhaps this is a more likely pathway than *via* $ONOO^-$ formation.[292]

The inefficiency of this pathway would account in part for the high concentrations of $^{\cdot}NO$ required for nitration to occur. However, whether $^{\cdot}NO$ or $ONOO^-$ is used in nitration experiments, protein and low molecular weight thiols will be oxidised more easily than tyrosine will be nitrated. In these experiments, nitrotyrosine formation did not occur until thiols were substantially depleted, by at least 40% (Figure 6.6). A study of BSA oxidation by $ONOO^-$ reports that at 1:5 molar ratio BSA:$ONOO^-$ 74% of cysteine residues were oxidised, while only 1.4% of tyrosine residues were nitrated.[104]

6.4.2.2 *In vivo* Nitrotyrosine Formation

Preliminary experiments detecting nitrotyrosine by western blotting of mucosal homogenates from control and DSS-exposed mice, and IBD biopsies revealed no increase in the anti-nitrotyrosine signal from inflamed compared to non-inflamed tissue samples (Figures 6.7 and 6.8). This suggests that nitrotyrosine formation is not a major player in the tissue damage of DSS-induced colitis and IBD. *

This finding in DSS-induced colitis again contrasts TNBS-induced ileitis, where an immunohistochemical study found nitrotyrosine immunoreactivity colocalised with iNOS expression and was evident throughout the gut wall, especially in the epithelium and enteric neurons.[172] However, this is consistent with the lack of increase in iNOS activity and the absence of disease improvement with NOS inhibition (Section 6.4.1). Consideration of the extent of thiol depletion in this model, a 26% decrease in reduced thiols including low molecular weight thiols, and the lack of difference in protein thiols by [^{14}C]-IAM detection (Chapter 4), would also suggest that there may not be sufficient oxidation of protein thiols to allow substantial nitrotyrosine formation to occur. Within inflammatory foci, however, such as crypt abscesses, conditions may be sufficiently severe to overcome the scavenging capacity of reduced thiols, and allow nitrotyrosine formation to occur.

The lack of a role for $^{\cdot}NO$ in DSS colitis would be a point of deviation of this model from many other models of colitis, and, on the balance of evidence currently available, from the human disease, although the evidence of increased $^{\cdot}NO$ generation in IBD is largely indirect and its role in pathogenesis has not been defined. Further, there may be differences in the involvment of $^{\cdot}NO$ in acute and chronic inflammation, however our current understanding of chronic and acute DSS-induced colitis does not easily fit the prevailing explanation. Mechanisms of disease peculiar to DSS-induced colitis may be involved, such as the toxicity of DSS to epithelial cells or other components of the mucosa involved in $^{\cdot}NO$ production.[48,172]

The lack of difference in nitrotyrosine signal by western blotting between inflamed and non-inflamed IBD biopsies is also contrary to brief reports of immunohistochemical localisation of iNOS and nitrotyrosine immunoreactivity with macrophages and neutrophils of the inflamed mucosa of IBD.[311,312] The concentration of nitrotyrosine around inflammatory cells may be important in achieving detectable quantities of the

*Controls using nitrotyrosine competition or second antibody only are required to ensure that antibody binding is specific.

modified proteins. Localised increases in oxidised proteins, once distributed across the whole tissue by homogenising, may not result in sufficient signal to be detected by western blotting, the tradeoff made for the potential gain of being able to identify oxidation of specific proteins. Furthermore, the potential rapid degradation of oxidised proteins within inflammatory foci may prevent accumulation of such indicators of tissue injury, although smearing of protein bands and associated nitrotyrosine signal would be expected in this case.

6.4.3 Conclusion

The lack of efficacy of NOS inhibition and the absence of $ONOO^-$-modified proteins does not support a role for excessive $^{\cdot}NO$ production in the pathogenesis and tissue injury of DSS-induced colitis. Similarly, these studies did not find increased nitrotyrosine-modified proteins in IBD. The contrast between these findings and other models of intestinal inflammation are reflective of the complex role of $^{\cdot}NO$ in intestinal function, which is yet to be fully elucidated.

Chapter 7

Concluding Discussion and Future Directions

7.1 The Aims and Achievements of this Thesis

IBD is an idiopathic disorder for which there is substantial indirect evidence suggesting the involvement of RONS in its pathogenesis. Many advances in the understandig of IBD pathogenesis have come from animal models of IBD which offer relief from the difficulty of obtaining clinical samples for research. Induced models of colitis are appropriate for examining mucosal tissue injury, for while the initiating events are different to IBD, it is likely that there are common mechanisms of tissue injury in the later stages of disease. This thesis undertook to find evidence to support the hypothesis that oxidative tissue injury was involved in the pathogenesis of DSS-induced colitis in mice, and to explore how this related to IBD.

Towards this, a variety of markers of oxidant injury, which had been previously investigated in IBD, were examined in the mucosal tissue of mice with acute colitis induced by administering 5% DSS in their drinking water for 7–11 days. Increases in MPO activity of 3–10-fold were found in the inflamed mucosa, representing the potential for increased oxidant production by neutrophils in the mucosa. The altered antioxidant status, with decreased total scavenging capacity, depletion of ascorbate and ubiquinol, and increased levels of α-tocopherol and urate, was consistent with excessive oxidant production within the tissue. The decrease in total reduced thiol levels and GAPDH activity are also supportive of oxidative stress.

This evidence supports the hypothesis that RONS are mediating tissue damage in DSS-induced colitis. The changes observed closely resemble those reported in human IBD tissue, indicating that DSS-induced colitis is a highly suitable model to pursue further investigations into the molecular basis of the oxidative tissue injury in IBD.

The second thrust of the investigations of this thesis concerned strategies to identify direct evidence of oxidative tissue injury in DSS-induced colitis and where possible, to apply these strategies to IBD samples.

Evidence of increased oxidant production was sought in mice using the salicylate hydroxylation method of detecting $^{\bullet}OH$. However, this method was not informative in the context of DSS-induced colitis because of the extent of changes in the physiol-

ogy of the diseased animals. Examination of protein carbonyls in DSS-induced colitis found that while total levels were not elevated, specific proteins may have been oxidised in this manner. The opportunity now exists to identify these proteins, and assess the potential contribution of their oxidation to the disease. Preliminary experiments with IBD tissue found that the levels of carbonyl groups were not consistently elevated in inflamed IBD mucosa, however, a similar profile of proteins containing carbonyls was observed, suggesting that further investigations in the mouse may be relevant to the human disease. Evidence of protein modification specific to oxidation by the $^{\bullet}NO$-derived peroxynitrite, was also sought by performing western blotting on mucosal homogenates for nitrotyrosine, however, no evidence of nitration of proteins in inflamed mucosa from DSS-exposed mice or IBD was found. The role of $^{\bullet}NO$ in DSS-induced colitis was also investigated by inhibition of $^{\bullet}NO$ synthesis with L-NAME, but this also failed to reveal a clear role for $^{\bullet}NO$ in this disease.

Numerous markers of oxidative injury were present in the DSS model of colitis and were consistent with the human disease. This is the first report on the involvement of oxidants in DSS-induced colitis, and few models of experimental colitis have been characterised in this manner. Attempts to provide further evidence of oxidant involvement in this disease were not always fruitful, but it should not be concluded that oxidants are not involved or not important, as there are many different approaches that may be taken and methodological problems to overcome when dealing with oxidative tissue injury in a diseased tissue.

7.2 Approaches to Oxidative Injury in Disease

The focus of this thesis was to provide evidence of oxidant injury in the inflamed mucosa of mice with DSS-induced colitis. To determine whether RONS make a significant contribution to the pathology of a disease, the following need to be demonstrated:[115]

- RONS are formed at the injury site.
- The time-course of their formation is such that they could play a role in disease pathology.
- Removal of RONS or prevention of their formation has beneficial effects.
- Direct application of RONS at concentrations found *in vivo* reproduces most or all of the injury.

Further, if the possibility of antioxidant therapy is being considered, then more detailed questions arise as to:

- What biomolecule is the compound supposed to protect?
- What oxidant is the compound protecting from?
- How is it being protected — by scavenging or by preventing oxidant production?

- Will the compound be present *in vivo* in the appropriate location and at sufficient concentrations to mediate the desired effect?

These questions are not easy to answer. They require accurate assay methods for oxidants, which are short lived and difficult to measure, and for the molecular targets, which are many and varied, depending on the nature of the oxidative stress — the RONS generated, the cell target, the antioxidant defences, and the presence or absence of transition metal ions.[115]

Appropriate methodology has limited our understanding of the role played by RONS in disease pathology. However, investigations of RONS in disease, with an outlook for potential antioxidant therapy, are of particular importance in diseases where current therapeutic aproaches are inadequate.[115] The work in this thesis has addressed some of these different approaches. While more techniques are becoming available for the study of oxidants and oxidative tissue injury in disease, those approaches and techniques most relevant to IBD are discussed below in light of the experience gained during this project.

7.2.1 Increased Oxidant Production

Oxidants are by nature very elusive. They are often short-lived and are removed rapidly by antioxidant enzymes. In this thesis evidence of increased production of oxidants was sought by measuring MPO levels (which are indicative of the changing potential for HOCl generation) and by trapping $^{\cdot}OH$ with salicylate hydroxylation. The salicylate hydroxylation method, while it has provided good evidence of increased $^{\cdot}OH$ generation in other diseases[203] was, unfortunately, uninformative in DSS-induced colitis. While MPO levels were found to be elevated by 3–10 fold, it should not be concluded that HOCl production was elevated, as that is dependent on substrate availability. Thus it is desirable to perform further experiments to examine more closely the question of oxidant production in the inflamed mucosa of DSS-induced colitis.

Assessment of the chemiluminescence of inflamed and non-inflamed mucosa is one method which could be readily applied to the DSS model, as it has already been used in other models of colitis and IBD to demonstrate increased production of oxidants.[128,130] This would give an indication of total RONS production and, combined with scavengers, some indication of the relative amounts of different oxidants being produced. An alternative approach to detecting radicals is electron spin resonance spectroscopy (ESR). This technique detects energy changes that occur as unpaired electrons align in response to an external magnetic field.[313] As the biologically relevant radicals do not reach sufficient concentrations to be detected directly, they must be "trapped" by molecules known as spin traps, which form more stable radicals that are then detected by ESR. The use of different spin traps allows different radicals to be specified. However, as systemic administration of the spin trap would be necesary to capture the radicals *in vivo*, the problems of variation in tissue distribution may be encountered, as was the case with salicylate, an inherent problem with the use of any exogenous "trapping" molecules. Further, ESR cannot be used for the measurement

of non-radical oxidants, such as HOCl and H_2O_2, which may be major contributors to oxidant stress in inflammatory diseases such as colitis.

From a different perspective, several methods used for measurement of oxidant production *in vitro* have now been adapted for use *in vivo* in a histochemical approach to the detection of oxidants. If these methods can be successfully applied to a range of tissues in various disease states, the amount of information to be gained is enormous when compared to present techniques. The ability to observe oxidant production *in situ* would allow the location of oxidant stress, the identification of candidate source and target cells or structures, and the direct association of morphological changes in the tissue with oxidant detection. Oxidant production may still be quantified using image analysis and the problem of "dilution factors", in conditions such as colitis where the tissue is not uniformly diseased, would be overcome.

At present, there are three methods for histochemical assessment of oxidant production. Cerium chloride forms an electron dense precipitate upon reaction with H_2O_2, which can be observed by electron microscopy.[314] This method has been applied to investigation of H_2O_2 production within various tissues, including smoke-exposed tracheal explants and ischaemic heart.[315–317] 2′,7′-Dichlorofluorescin diacetate can detect intracellular H_2O_2 and $^{\bullet}NO$,[318,319] and has recently been used for examining oxidant production in ischaemic lung injury.[320] The tissue is perfused with the dye, which is trapped inside the cells after hydrolysis by esterases and becomes fluorescent after oxidation. Fixed tissue is then examined by fluorescence confocal microscopy.[321] The third method utilizes the oxidation of diaminobenzidine to form amber-coloured osmiophilic polymers, which can be observed by light or electron microscopy. Perfusion of organs with diaminobenzidine in combination with Mn^{2+} or Fe^{2+} will allow detection of $O_2^{\bullet-}$ or H_2O_2 production respectively.[322] At this stage these methods have mainly been applied to perfused animal tissues subjected to ischaemia / reperfusion, however, the potential to employ them in investigations of experimental colitis and adapt them for use on explant human tissue may provide a much greater understanding of the role of oxidant production in the pathogenesis of this disease.

7.2.2 Evidence of Oxidant Attack on Biomolecules

Previous studies in IBD have examined antioxidant levels, lipid peroxidation and also GAPDH activity in inflamed and non-inflamed tissue to demonstrate the occurrence of oxidative tissue injury in the inflamed mucosa.[141,142,149,150] In this thesis, similar indicators were examined in DSS-induced colitis, with changes in the levels of specific antioxidants or decreases in a specific enzyme activity being identified. Overall indicators of oxidant injury were also measured, such as the total scavenging capacity and total reduced thiol content. When looking for new indicators of oxidant injury in colitis, both overall and specific methods were applied where possible (as in the analysis of carbonyl groups) in an attempt to identify specific defects in the mucosa which may have pathological consequences.

As was the case for oxidant detection, an alternative approach is to examine ox-

idant injury *in situ* using immunohistochemical techniques. Consideration of the location of oxidative events and targets within the diseased tissue may explain some of the variation experienced in these studies in the ease of detection of different indicators of oxidative injury. In DSS-induced colitis, the whole tissue is not uniformly diseased, as ulcers and inflammatory foci form which may be surrounded by less intensely involved tissue (Figure 2.3). Within inflammatory foci, oxidant concentration is potentially high and may lead to events such as antioxidant depletion and thiol oxidation, which in turn may make it possible for "later" oxidative events such as carbonyl and nitrotyrosine formation to occur. Outside these areas, the oxidant stress may only be sufficient to deplete the highly susceptible targets (such as antioxidants and thiols), or movement of antioxidants or thiol exchange reactions may transfer these targets into the intensely involved areas of tissue to replenish those depleted by the high oxidant concentrations. Thus susceptible targets such as antioxidants and thiols may be decreased on average across the whole tissue and thus readily detected biochemically, while evidence such as carbonyl and nitrotyrosine formation is restricted to the inflammatory foci. Furthermore, increased protease activity within the foci, especially if directed at oxidised proteins (as can occur in response to oxidative stress[274]) may mean that markers such as carbonyl and nitrotyrosine groups are only present transiently and unable to accumulate, thus further increasing the difficulty of detecting them biochemically. The problem of uneven distribution of disease is highlighted by contrasting the regional staining for nitrotyrosine observed in TNBS-induced ileitis and IBD[172,311] with the more uniform occurrence of nitrotyrosine in, for instance, ischaemic or acute lung injury, where intense nitrotyrosine staining was observed throughout the lung.[308,310]

The availability of tools for an *in situ* approach to oxidative injury have been limited. While carbonyl groups can be observed with antibodies by western blotting after reaction with DNPH, the need to remove DNA, the non-specific nature of this group and the potential for carbonyls to react or to be formed during fixation procedures must be considered before this reaction could be used in immunohistochemistry. Of greater interest is the recent report of antibodies developed against HOCl-oxidised low density lipoprotein.[323] Among those produced, monoclonals were selected that reacted with HOCl-modified albumins as well as lipoprotein, suggesting that this antibody recognized epitopes that are commonly generated on proteins exposed to HOCl. These antibodies have been used to identify the presence of HOCl-modified proteins within atherosclerotic plaques.[324] Such a tool may prove very useful in investigating the role of HOCl-mediated damage to proteins in inflammatory diseases, and could readily be applied to DSS-induced colitis and IBD. The study of oxidative tissue injury would benefit enormously from the further development of markers which can be examined *in situ*.

In terms of new biochemical indicators of protein oxidation, other reaction products of tyrosine may serve as useful indicators. The chlorination of tyrosine to form chlorotyrosine occurs in peptides or proteins exposed to HOCl or MPO and is being developed as a specific marker for the production of HOCl *in vivo* and for the involvement of myeloperoxidase in inflammatory tissue damage.[325,326] Dityrosine formation

can also be measured as an indicator of protein oxidation.[228,327] As discussed in Chapter 3, the dimerization of phenolics is not specific for a given oxidant, but may provide an overall indicator of protein oxidation, with more specificity and sensitivity than the measurement of protein carbonyl groups. It is presently used in *in vitro* work as an indicator of protein oxidation,[229,328] but for both dityrosine and chlorotyrosine, they are yet to be applied to whole and diseased tissue, the transition from *in vitro* to *in vivo* work always being the difficult step. As with nitrotyrosine, the potential exists for the development of antibodies against chlorotyrosine and dityrosine.

7.2.3 Intervention Investigations

Intervention studies have been performed on other models of colitis to provide evidence of oxidant involvement in the disease process, with a wide range of scavengers, desferrioxamine, inhibitors of $^{\bullet}NO$ production, and a GSH sparing agent reducing the severity of disease (Section 1.3.4). In this thesis, the effect of inhibiting NOS activity on DSS-induced colitis was investigated. While this strategy can provide further support for the role of oxidants in the pathogenesis of disease, it does not necessarily implicate oxidative tissue injury. Most of the studies in experimental colitis examining the effects of antioxidant intervention have measured outcomes in terms of general disease indicators such as histological scoring, symptoms, and mucosal permeability. While this is valid from a clinical perspective, it provides no information with respect to the mechanism of action of the treatment.

Oxidants may be involved in inflammation through several mechanisms other than the direct mediation of tissue injury (Section 7.5), therefore the precise mechanism remains unknown unless the use of a scavenger is combined with measurement of oxidant production, oxidative tissue injury markers or other indicators. Investigations of the inhibition of $^{\bullet}NO$ synthesis in DSS-induced colitis highlight this problem, where inhibition of the vascular functions of $^{\bullet}NO$ versus the cytotoxic capabilities of $^{\bullet}NO$ may have very different effects on disease (Section 6.4). While the development of inhibitors specific to different NOS isoforms has helped separate these functions of $^{\bullet}NO$, the understanding gained by a "combination approach" is illustrated by the work of Miller and collegues on TNBS-induced ileitis, where increased $^{\bullet}NO$ production and nitrotyrosine formation in disease, and amelioration of disease by NOS inhibition have provided a good understanding of the role of $^{\bullet}NO$ in this disease.[135,172]

The effects of scavengers such as SOD-mimics and $^{\bullet}OH$ scavengers are yet to be tested on DSS-induced colitis. Having now established a range of possible markers of oxidative stress in the inflamed mucosa, they can be assessed in both clinical and oxidative injury outcomes. In addition, these markers may be used to help better understand the actions of current IBD treatments, such as 5-ASA (which is effective in DSS-colitis), in terms of their antioxidant capacities *in vivo* in experimental colitis.

7.2.4 Application of Molecular Approaches to Oxidant Injury

While a nucleic acid-based approach is proving popular and informative in many forms of disease, at present there is little opportunity for application of these techniques to the study of oxidative injury, as the production of oxidants is rarely regulated at the level of gene expression. In neutrophils, oxidant production is increased in response to stimuli by assembly of the pre-formed subunits of the NADPH oxidase for production of $O_2^{\cdot-}$ and by release of MPO from the neutrophilic granules. $^{\cdot}OH$ generation is limited by the availability of free iron, and iron binding proteins are normally are well below saturation, and can thus accommodate increased iron levels without protein synthesis.

$^{\cdot}NO$ is an exception to these problems, and there is rapid expansion of the literature on the molecular basis of $^{\cdot}NO$ synthesis, different isoforms of NOS and the regulation of their expression. iNOS gene expression has been investigated in TNBS-ileitis and in rhesus macaques by reverse transcription-polymerase chain reaction, where iNOS RNA was found only in mucosa from animals with colitis, and not in control animals.[48,172]

Antioxidant enzymes are also candidates for the molecular approach. The expression of manganese and copper/zinc SOD has been examined in the induction of colitis with acetic acid.[145] Both mRNA and protein expression of Mg-SOD, but not CuZn-SOD, in the colon were increased by more than 10-fold in the first 24 hours after luminal exposure to acetic acid, suggesting that induction of MnSOD in specific cell types may have a cytoprotective function. A recent report has combined examination of iNOS and SOD expression in TNBS colitis in rats to show that iNOS expression is elevated during colitis and SOD activity was decreased (although mRNA levels were elevated), a situation which would favour $ONOO^-$ formation and enhance tissue damage in this model.[144]

7.3 Focus on the Importance of Neutrophils in this Disease

The importance of neutrophils in DSS-induced colitis has been demonstrated by the reduced severity of disease in neutrophil-depleted mice,[179] while the recruitment of neutrophils into the active lesion and the producion of neutrophilic oxidants in the inflamed mucosa implicate neutrophils in the pathogenesis of IBD.[18,20,128,130] Future work would thus be well directed if it focussed on the role of neutrophil-mediated oxidant injury. The approach taken in this thesis was not specifically aimed at neutrophil-mediated tissue injury — there is no definitive proof for $^{\cdot}OH$ production by neutrophils,[329] and $^{\cdot}NO$ is appears not to be of primary importance in the bactericidal activity of neutrophils.[71,92,330] The components thought to be most central to the toxic potential of neutrophils are the production of HOCl by MPO, and release of proteinases from the neutrophilic granules.[28]

Tools specific for MPO/ HOCl-mediated tissue injury have not been readily available, but several newly developed methods on this theme have already been mentioned. The measurement of chlorotyrosine and the use of antibodies to HOCl-mod-

ified proteins will contribute to elucidating the role of neutrophils and HOCl in the mediation of oxidative tissue injury in inflammatory diseases. Specific inhibitors of MPO would also be an important tool, but while many compounds are available which inhibit MPO, including many non-steroidal anti-inflammatory drugs,[119] these have a multitude of other effects. Specific MPO inhibitors are being developed, such as 4-aminobenzoic acid hydrazide and salicylhydroxamic acid, but at this stage have only been used *in vitro*.[331,332] Alternatively, scavengers such as taurine and methionine may be used to intervene in HOCl-mediated injury.

The relationship between neutrophil-derived proteinases and oxidants in the destructive capacity of neutrophils should not be forgotten. Neutrophil-derived HOCl inactivates cellular/ extracellular α_1-antiproteinase, preventing inhibition of elastase, and activates the latent collagenase and gelatinase released from neutrophil granules.[28] These enzymes amplify the destructive potential of oxidants, as one enzyme molecule can cause more degradation than one molecule of oxidant acting directly.

In IBD, interest has been shown in neutrophil proteinases from a diagnostic perspective with the finding of correlations between plasma or faecal assessment of elastase/ α_1-antiproteinase levels and disease activity.[333–335] However, while neutrophil proteinases have been investigated in many inflammatory diseases,[28] only collagenase and stromelysin have been measured in the inflamed and non-inflamed intestinal tissue from IBD patients.[336,337] The elevation of plasma and faecal neutrophil elastase in IBD patients, and the ability of 5-ASA to protect α_1-antiproteinase from inactivation by neutrophils *in vitro*[166] begs the question of what role this destructive protease may be having in the mucosa, and the role that oxidation may play in the activity of elastase and other neutrophil proteinases.

Investigations of this nature have been performed on two diseases of the lung. In adult respiratory distress syndrome, bronchoalveolar lavage fluid was found to contain inactivated α_1-antiproteinase, activity of which could be restored by reduction of the oxidised methionyl residue.[338] Elastase/elastase-inhibitor imbalance has also been implicated in the pathogenesis of pulmonary emphysema, where studies have indicated that α_1-antiproteinase recovered from smokers lungs has only half of the predicted normal activity per mg inhibitor and contains 4 moles of methionine sulfoxide (oxidised methionine) per mole of inactive inhibitor, while non-smokers lungs contained only native α_1-antiproteinase.[339] Studies like these have led to the investigating synthetic inhibitors of elastase which are resistant to oxidative inactivation,[340] to help ameilorate the damage due to smoking observed in the lung. As in the lung, there is the opportunity in the colon to use such agents topically, delivering them to the site of action of these enzymes, where they may reduce tissue damage or perhaps assist regeneration of the tissue. However, in investigating these aspects of colitis in experimental colitis, potential species differences in the oxidative susceptibility of these inhibitors and of the neutrophil proteinase profile must be kept in mind.[28]

7.4 Oxidative Injury and Pathogenesis of Colitis

The primary aim of this work was to identify markers of oxidant injury which may be present in the diseased tissue of DSS-induced colitis; linking oxidants with the disease process is another issue. Having established the presence of several markers of oxidative injury in DSS-induced colitis, there is now the opportunity to explore how they may be associated with the pathogenesis of disease, looking at changes with time-course of disease, with the arrival of oxidant-producing inflammatory cells and morphological changes in the tissue.

The levels of ascorbate, urate, α-tocopherol and ubiquinol were all found to be significantly altered in DSS-induced colitis (Chapter 4). As discussed in Chapter 4, the large decrease in ubiquinol-9 content may be detrimental to mitochondrial function, so assessment of mitochondrial function in the mucosa in relation to disease may be of interest.

Thiols are very susceptible targets of oxidants, and are oxidised readily, if not preferentially, by most oxidants.[103,236,290,308,341] This susceptibility together with the decreased GAPDH activity and total reduced thiol content in DSS-induced colitis (Chapter 4) and the importance of thiols in intestinal function (Section 4.4) calls for a closer examination of the role of thiols in DSS-induced colitis. In addition, as discussed in Chapter 4, thiol oxidation may be reversible. Analysis of changes in mucosal and plasma levels of GSH, GSSG, protein-GSH disulfides, and the reversibility of inactivation of thiol-containing proteins such as GAPDH and Na/K-ATPase, over the course of disease and recovery would offer invaluable information towards an understanding of the role of thiol oxidation in colitis, and of the potential for thiol-reducing compounds as therapeutic agents in colitis.

The examination of protein carbonyl groups in DSS-induced colitis revealed several mucosal proteins which may be particularly susceptible to carbonyl formation (Chapter 5). Establishing the identity of these proteins would allow assessment of their activity and function in normal and inflamed mucosa, in both human and mouse tissue, to determine whether carbonyl oxidation is interfering in mucosal function during disease. The role of iron in mediating this oxidation may also be further investigated with the prospect of chelators being able to reduce the amount of damage *via* this mechanism.

7.5 Indirect Involvement of Oxidants in Inflammation

The discussion thus far has dealt primarily with tissue injury mediated directly by RONS through the oxidative modification of key biomolecules. However, there are other indirect ways in which oxidants may influence tissue injury and the inflammatory response. The physiological effects of oxidants are being increasingly recognised, especially as the many functions of $^{\bullet}NO$ have been appreciated. These mechanisms provide the potential for an oxidant / antioxidant imbalance to lead to greater tissue injury than *via* direct oxidation.

As described in detail in Section 7.3, oxidants can alter the neutrophil proteinase / anti-proteinase balance of a tissue, thus leading to degradation of the extracellular matrix by elastase, collagenase and gelatinase. $^{\cdot}NO$ also has many effects on blood flow and platelet function and nervous signals[71] which are all potentially involved in the antiinflammatory activity of NOS inhibitors in models of intestinal inflammation.

Oxidants can modulate neutrophil-endothelial cell interactions, thus affecting adhesion and consequent migration of neutrophils during acute inflammation. Oxidants such as $O_2^{\cdot-}$, H_2O_2, and monochloramine are pro-adhesive[90,342] while inhibition of $^{\cdot}NO$ synthesis is pro-adhesive, possibly by reducing $O_2^{\cdot-}$ scavenging by $^{\cdot}NO$.[303,343] The pro-adhesive effects are in part mediated through activation of the adhesive glycoprotein CD11/CD18 on the surface of neutrophils, and can be inhibited by scavengers, such as SOD and catalase, by $^{\cdot}NO$ donors, and by anti-CD18 antibodies.[90,303,342]

In addition to modulating the ability of leukocytes to migrate, oxidants have also been shown to stimulate the expression of cytokines by various cell types. Low concentrations of H_2O_2 can stimulate the production of interleukin 8 by whole blood, hepatocytes and skin fibroblasts,[344] and macrophage inflammatory protein 2 (MIP-2) production by lung macrophages[345] is also stimulated by H_2O_2. Further, antioxidants such as dimethylsulfoxide, a $^{\cdot}OH$ scavenger, could inhibit interleukin 8 production by whole blood stimulated with LPS or immune complexes.[344] Macrophage expression of macrophage inflammatory protein-1α (MIP-1α) has also been induced by H_2O_2 and menadione, each oxidant involving a different mechanism — H_2O_2 by increasing the half life of the mRNA, and menadione by increasing the rate of transcription of MIP-1α RNA.[346] Thus low levels of RONS may perpetuate the inflammation in chronic diseases by stimulating the production of chemotactic peptides which then recruit neutrophils to sites of inflammation.

Effects like these are likely to be mediated *via* intracellular signalling events in which oxidants are involved. At least two well-defined transcription factors, nuclear factor κ B (NFκB) and activator protein 1 (AP-1) have been identified as being regulated by the intracellular redox state, particularly by the GSH-GSSG balance.[67] In the immune system, T cell activities have been demonstrated to be modulated by redox status and NFκB activity, with T cell responses to CD4, CD8 and interleukin 2 receptor ligands being sensitive to both increases and decreases in intracellular GSSG concentration.[347] Further, in the intestine, the antigen receptor-dependent activation pathway of lamina propria T lymphocytes for proliferation is down-regulated by intestinal mucosa derived factor(s),[348] which have been found to be small, nonprotein, nonpeptide molecules with oxidative properties.[349] Thus, modulation of lamina propria T cell responsiveness is another means by which an imbalance in the redox status of the mucosa may contribute to the inflammatory response.

7.6 Conclusions

The above discussion illustrates the complexity of the potential involvement of RONS in IBD, and direct mediation of tissue injury may be just one of many aspects of their role in IBD. Despite this complexity, examination of the molecular basis of tissue injury within the active lesion is an appropriate focus for investigation. The characterisation of experimental models of colitis is an important step in the progress of our understanding.

This work has contributed to the understanding of oxidative processes in the inflamed mucosa of acute DSS-induced colitis and IBD. In doing so, it has opened opportunities to obtain a clearer picture of the tissue injury processes in DSS-induced colitis, and through this model, provide insights into the possibilities for new therapeutic strategies for IBD.

Appendix A

Disease Course of DSS-Induced Colitis

The acute colitis induced in mice by administration of DSS is described in detail in Chapter 2. Figure A.1 shows the disease course of the mice whose tissue was used in the experiments of this thesis, and whose disease course has not been reported elsewhere. Table A.1 contains details of the data in Figure A.1.

Some variation was experienced in the length of exposure to 5% DSS required to induce disease of sufficient severity for these studies (Figure A.2). Mice were maintained on 5% DSS until diarrhoea was seen in at least 80% of animals and body weights were no greater than initial weights, while avoiding deaths from disease. Symptoms first appeared between 2 and 4 days of 5% DSS exposure, and the length of exposure ranged from 7–11 days. The rate of weight gain in control mice was also variable, and was related to the inital weight. In experiment A, only initial and final weights were noted: control mice weights increased by 14% while the final weight of DSS-exposed mice was less than 1% above mean initial weight (data not shown).

Set D is the outcome of 30 mice which were divided into 3 groups for use in the study of methionine on 2,3-DHB levels (Chapter 3). To demonstrate the consitency within a group of mice, the disease courses for these 3 groups of 10 mice and for the total number are shown in Figure A.2. The consistency of disease is most clearly visible in the weight change record (bottom panel, Figure A.2). The objective nature of weight loss monitoring is important to the consistence of this disease indicator. The assessment of diarrhoea and rectal bleeding, particularly in the early stages of the disease, is more subjective, and the grooming habits of the mice add further to the variation in diarrhoea and bleeding observed.

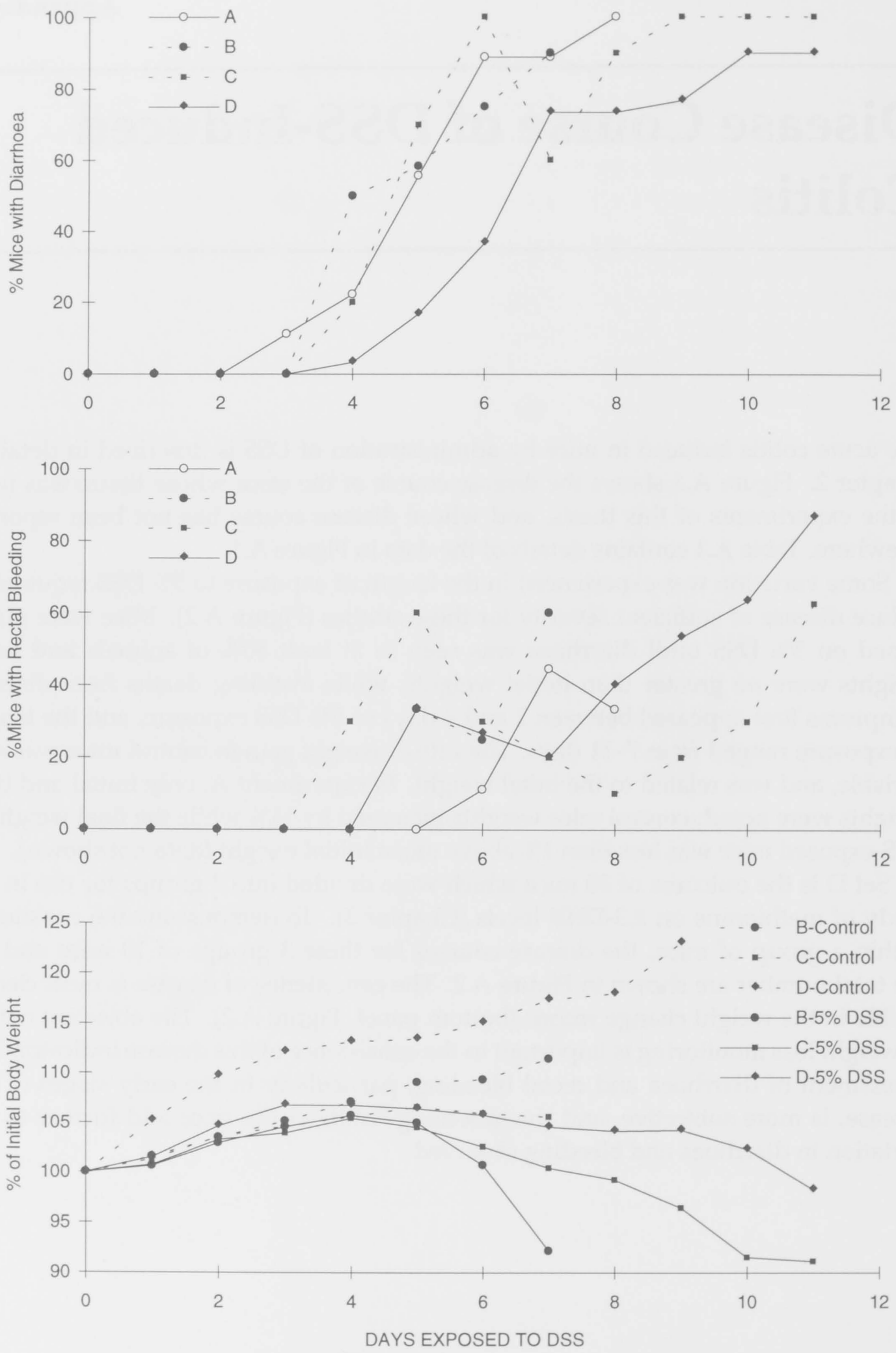


Figure A.1: Disease course of mice exposed to 5% DSS are displayed, showing percentage of mice observed with diarrhoea (top) and rectal bleeding (centre). Body weight has been expressed as % of initial weight (bottom). Details of the groups can be found in Table A.1.

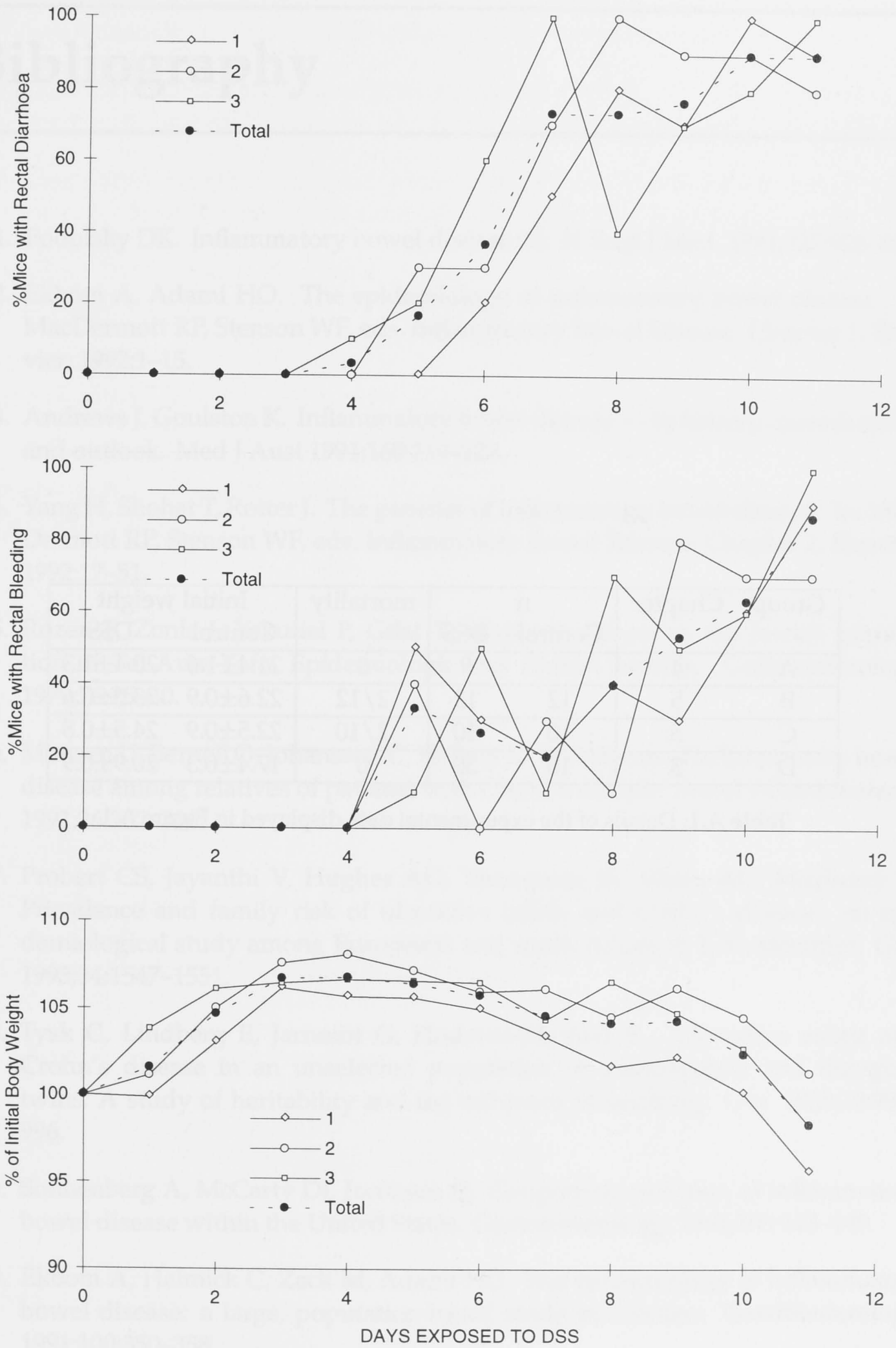


Figure A.2: Disease course of mice exposed 5% DSS. Symptoms of 3 groups of 10 mice from the same experiment and the mean of the 3 groups are displayed.

Group	Chapter	n		mortality	Initial weight	
		Control	DSS		Control	DSS
A	4	9	9	0	21.4±1.6	20.4±1.3
B	5	12	12	2/12	22.6±0.9	23.0±0.6
C	5	10	10	2/10	22.5±0.9	24.5±0.8
D	3	10	30	0	17.4±0.3	20.9±0.9

Table A.1: Details of the experimental data displayed in Figure A.1.

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